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Results are summarized from the analysis of ER-α splicing variants in breast tumor cells. A highly diverse population of variants were observed representing primarily exon-skipped transcripts, but also including novel variants resulting from promiscuous or cryptic splicing. Techniques and reagents suited for the analysis of ER-α splicing variants were developed and described. Splicing variants were observed to be much more prevalent for ER-α than for most other genes including the progesterone receptor gene. The biochemical properties and transcriptional activities of the major ER-α splicing variants were also characterized. Two variants (ERΔE3 & ERΔE5) were identified that share significant residual function with intact ER-α. These properties confer on the ERΔE3 & ERΔE5 receptor variants the ability to inhibit the activity of some genes, but to stimulate the transcription of other genes. Genes that represent targets for induction by these ER-α splicing variants appear to lack consensus DNA-binding sites for ER-α, but instead they are regulated indirectly through interactions with other transcription factors such as AP-1. ER-α splicing variants thus function primarily through a recently proposed non-classical pathway for estrogen action. A variety of potential gene targets for regulation by the ERΔE3 & ERΔE5 variants are described.

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Richard J. Miksicek 6/22/00

PI - Signature **Date**

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INTRODUCTION

Title: Splicing Variants of the Estrogen Receptor in Breast Cancer
ID No.: DAMD-1794-J-4372
PI: Richard J. Miksicek
Period: 10/01/98 through 09/30/99 (final year)
10/01/94 through 09/30/99 (all years)

Summary of Administrative Matters

This USAMRMC Breast Cancer Research Award was made to Richard J. Miksicek, who holds an appointment as Assistant Professor of Physiology and Member of the Cancer Center at Michigan State University (MSU). This report summarizes the entire five year period of this award, with an emphasis on the final year (10/1/98-9/30/99) not covered in previous reports. At the time this grant was awarded, Dr. Miksicek was on the faculty at the State University of New York (SUNY) at Stony Brook. During the first year of this award, the P.I. accepted a new position (effective 07/01/95) in the Department of Physiology at MSU. At that time, permission was requested from the US Army MRMC through the awardee institution (The Research Foundation of SUNY) to effectively change the site of performance of this project from SUNY @ Stony Brook to MSU by establishing a research subcontract between these institutions. This research subcontract stipulated that Dr. Sandra Haslam (Professor of Physiology and Director of the MSU Breast Cancer Program) be named as Principal Investigator for the MSU subgrant with Dr. Miksicek continuing to serve as P.I. on the primary award to SUNY @ Stony Brook. Throughout the entire award period, Dr. Miksicek has been acting as Principal Co-Investigator at MSU with primary responsibility for the conduct of research and grant administration. This subcontract was accepted by both institutions on 03/28/96 and became effective retroactively to 07/01/95. Progress on this project was significantly delayed between 07/01/95 and 03/28/96 pending negotiation of the research subcontract and release of research funds at MSU. During 1996, the investigator's laboratory was re-established at MSU and new research staff were recruited to resume work on this project. Personnel who contributed to this project and their periods of service are provided, below:

Key Personnel:

Name	Degree	Role	Effort	Period of Service
MIKSICEK, Richard	Ph.D.	PI (SUNY) & Co-PI (MSU)	25%	09/94-09/99
ANKRAPP, David	Ph.D.	Postdoctoral Research Fellow	100%	07/96-09/98
BOLLIG, Aliccia	B.S.	Graduate Research Asst.	100%	10/95-09/99
MORRISON, Mary	M.S.	Research Asst.	15%	02/96-03/98

In addition, this project provided research experience for two rotating graduate students at MSU (Maria Pino & Lori Swenson) and six MSU undergraduate assistants (Andrea Alexander, Jennifer Garner, John Hicks, Khaled Ismail, Brian Markle, & Carrie Preston).

In Fall 1998, the PI requested a one year no-cost extension through SUNY at Stony Brook, the recipient of the primary award, in order to make up for time lost in moving this project from Stony Brook to MSU. During this final year, only the PI (Dr. Miksicek) and Ms. Bollig contributed effort to this project. Ms. Bollig drew partial salary support from this award during this time. She is currently supported by an individual predoctoral fellowship award (DAMD 1799-I-9293) related to a continuing aspect of this project.

Scope of the Project

Estrogen Receptor-alpha (ER-α)-positive human breast tumors and tumor-derived cell lines contain a mixed population of ER-α messenger RNAs (mRNAs), representing both correctly and aberrantly spliced transcripts (1-3). Since their discovery nearly ten years ago, these variant ER-α mRNAs were predicted to give rise to structurally altered receptor molecules with the potential to interfere with or modify the normal estrogen response pathway present in breast tumor cells. Early reports speculated that these variants might cause a loss of estrogen responsiveness or confer resistance to tamoxifen and other estrogen antagonists (4,5), thereby compromising the effectiveness of hormonal adjuvant therapy for breast cancer. A second important implication relates to the ability of ER-α splicing variants to distort the results of immunohistochemical tests (ER IHA) that are used clinically to determine the ER status of breast tumors, reducing the value of ER as a prognostic indicator in cancer management (6,7). We received this award in 1994 to undertake an analysis of splicing variants of ER-α mRNA in selected human breast tumor specimens and to characterize the functional activity of these variants. During the course of this project, we strove to refine analytical techniques and to develop reagents to enable further investigation of the clinical significance of these receptor variants.

RESULTS

Progress on this award is summarized separately under each task listed in the original project proposal, with greater attention given to information not yet published and not detailed in previous reports. Experimental methods used were as described in previous reports or publications.

Task 1, Identification of ER mRNA variants in breast tissue (months 1-24):

a. Preparation of oligonucleotide primers for the analysis of ER mRNA splicing variants and optimization of PCR amplification conditions
b. PCR amplification of ER cDNAs prepared from tamoxifen-resistant tumors
c. PCR amplification of Progesterone Receptor (PR) cDNAs prepared from tamoxifen-resistant tumors
d. PCR amplification of ER cDNAs prepared from normal human breast tissue

As detailed in earlier reports, substantial effort was expended during years 2 and 3 to develop "streamlined" PCR-based assays for the analysis of ER splicing variants in breast tumor specimens and cell lines. This has proven to be a demanding task due to the considerable heterogeneity of ER-α mRNA splicing products that are encountered in estrogen-responsive tissues. This is further complicated by the large target size under analysis, since functionally relevant sequence variations can occur anywhere within the 2 kb reading frame of the 6.4 kb ER-α transcript. The primary analytical method proposed in the initial application and the one most extensively explored was "triple-primer" PCR amplification, in which a series of 3'-mismatched primers were designed to simultaneously co-amplify a single splicing variant along with wild type (wt) ER-α mRNA, thereby enabling a direct comparison of their relative abundance. A second approach that was extensively explored involved PCR amplification of the entire pool of ER-α variants, followed by "shot-gun" cloning into a sequencing vector. Initially, we attempted to assign variants by picking individual clones into microtitre plates, followed by replica plating onto nitrocellulose filters and hybridizing the resulting filters with a series of oligonucleotide probes designed to represent all of the possible correct and aberrant splice junctions. In practice, both triple-primer PCR and colony hybridization proved troublesome and, most importantly, failed to correctly assign 10-15% of the ER-α cDNAs in certain specimens due to the presence of unexpected or highly variable deletions. Ultimately, the only method which permitted the reliable identification of multiple exon deletion mutants and variants with non-canonical splice patterns, as well as the more common single exon deletion variants, consisted of targeted PCR amplification of ER-α sequences and the generation of a splice variant library by shot-gun cloning followed by direct DNA sequencing of individual clones. This method was therefore chosen for the analysis of ER-α (and PR) variants for the duration of the project.

This method was successfully employed during year 3 to analyze the profile of ER-α splicing variants in MCF7 cells and in a breast tumor with discordant ER status. During year 4 we also analyzed ER-α transcripts in two sublines of MCF10AT cells representing a spontaneously immortalized, but non-tumorigenic human breast epithelial cell line and in M13 SV1 cells (a second cell culture model for normal human breast epithelial cells). A detailed description of the results from these studies was provided in the corresponding annual reports and forms the basis of a manuscript in preparation. A similar analysis of ER mRNA splicing variants in tamoxifen-resistant tumors (Task 1b) was not completed as proposed since the necessary tissue specimens are very rare and could not be obtained for analysis. However, work relevant to this aim was conducted in a cell culture model for tamoxifen resistance (see below).

We failed to observe analogous splicing variants for progesterone receptor (PR) mRNA using PCR amplification (Task 1c), despite several reports in the literature describing PR variants lacking sequence corresponding to PR exons 2, 4, 5, or 6 (8-13). While PR splicing variants may exist in breast tissue, available evidence indicates that they are much less prevalent than splicing variants of the ER-α gene.

Identification of ERΔE3 as an abundant splicing variant in a tamoxifen-resistant cell line.

In experiments not foreseen at the time of the previous report, we identified ERΔE3 as representing a major isoform of ER-α in the tamoxifen-resistant cell line MCF-7/LCC2. LCC2 cells represent a stable population of the MCF-7 cell line selected for the ability to grow in the presence of 4-hydroxytamoxifen (14). While resistant to tamoxifen, they remain ER-positive, they respond to estradiol by a variety of criteria, and they are still growth inhibited by the pure estrogen antagonist ICI-182,780. In collaboration with the laboratory of Susan Conrad (Dept. of Microbiology, MSU) we observed that extracts from MCF-7/LCC2 cells contain a major ER-α variant estimated to be 55-60 kd in size by immunoblot analysis, in addition to the full-sized 67 kd receptor. Speculating that this was likely to correspond to an ER-α splicing variant, we undertook the characterization of this variant by PCR amplification of mRNA extracted from MCF-7/LCC2 cells. In contrast to DNA amplified in parallel from parental MCF-7 cells, the PCR products from LCC2 cells migrated on agarose gels as two distinct bands of approximately equal intensity. The upper and lower bands were excised for direct sequencing and were found to represent wt ER-α and ERΔE3, respectively. While minor variants are certainly also represented among the PCR amplified material, they were not detected due to the inability of direct sequencing of PCR products to detect all but the most abundant isoforms present.

The fact that wt ER-α and ERΔE3 could be detected in an approximately 1:1 ratio in MCF-7/ LCC2 cells suggested to us that these cells might be hemizygous for an allele of the ER-α gene containing mutated splicing signals flanking exon 3. However, PCR amplification of genomic DNA from MCF-7/LCC2 cells using intronic primers followed by direct sequence analysis failed to detect any mutations that might account for a failure to correctly splice exon 3. The molecular explanation for over-representation of ERΔE3 in MCF-7/LCC2 cells therefore remains undetermined. The consequences of ERΔE3 over-expression in MCF-7 cells with respect to estrogen responsiveness and tamoxifen sensitivity are the subject of continued study in collaboration with the Conrad laboratory and will be reported in the literature when these experiments are complete.

Task 1, Conclusions:

The major conclusions from the studies undertaken under Task 1 can be summarized as follows:

1) ER-α splicing variants invariably co-exist with wt ER-α mRNA in all ER-positive tissues and cell lines. The most abundant variant usually observed is ERΔE7.
2) In the typical pattern, ER-α variants lacking a single exon (exons 2, 3, 4, 5,or 7) are the most readily detected. Lower levels of variants lacking multiple exons, either in tandem or as separate internal deletions, are also observed.

3) In MCF-7 cells, variants harboring unusual internal deletions or insertions were also detected at a somewhat lower frequency (cumulatively representing approximately 10% of the transcript pool). These variants could be most easily rationalized as the products of non-canonical splicing events or splicing that utilized cryptic splice donor or acceptor sites.
4) Comparing the ER-α variants observed in a breast tumor cell line (such as MCF-7) with those from non-tumorigenic breast epithelial cell lines (such as MCF10A TG-1 and TG-3) suggests that the profile of variants becomes more heterogeneous as a result of breast tumor progression and includes more ER-α transcripts that contain either multiple exon deletions or unusual (non-canonical) splicing products.
5) In special circumstances, such as a breast tumor chosen for its discordant ER status (i.e., inconsistent immunohistochemical staining comparing two separate ER antibodies) or cells selected for tamoxifen resistance (i.e., MCF-7/LCC2 cells), one or a few splicing variants may exist as the predominant ER-α species.

Specific findings were as previously reported.

Task 2. Functional analysis of ER mRNA splicing variants (months 18-48):

a. Construction of ER expression plasmids harboring variant ER cDNAs.
b. Analysis of the transcriptional stimulatory or inhibitory activities of ER splicing variants by transient transfection.
c. Production and characterization of cell lines that stably express the "constitutive" ERΔE5 variant.

Work continued throughout the 5-year period of this project to characterize as fully as possible the biochemical and transcriptional activity of ER-α splicing variants. A paucity of functional information exists on the behavior of ER-α splicing variants and this project represents the most thorough analysis so far available. Functional analysis was limited to variants lacking a single internal coding exon, since these are predicted to be the most prevalent variants based on RNA analysis. Furthermore, the predicted reading frames of variants harboring multiple exon deletions are, with only a few exceptions, equivalent to the reading frames present in variants that lack a single exon. Much of our progress has been detailed in previous technical reports and in a recently published research report (see ref. 15, provided in Appendix A).

As commonly noted in the literature, splicing variants of ER-α are readily detected at the RNA level using techniques such as RNAse protection analysis and PCR amplification, but it has been more difficult to confirm that these variant mRNAs also give rise to stable receptor isoforms at the protein level. A common observation (and one shared by us) is that most ER-α antibodies, including those used in clinical assays whose specificity for ER-α is well documented, also detect minor immunoreactive species that differ in size from full-length ER-α. A representative immunoblot of MCF-7 cell lysates using an ERα-specific monoclonal antibody (Mab-17) developed in this laboratory is shown in Fig. 1. This antibody recognizes an amino-terminal epitope shared by virtually all ER-α splicing variants and shows no cross-reactivity with proteins present in ER-negative cell lines (16). Wt ER-α (67 kd) is the most prevalent band in MCF-7 whole cell (WCE) or nuclear extracts, consistent with the relative abundance of correctly processed mRNA as determined by RNA analysis. Of note, the Mab-17 antibody (similar to other ER-α antibodies such as H222, H226, D75, and ER 1D5) also detects a variety of additional polypeptides in MCF-7 cell lysates, varying in size from 20-85 kd. Paralleling what we and others observe

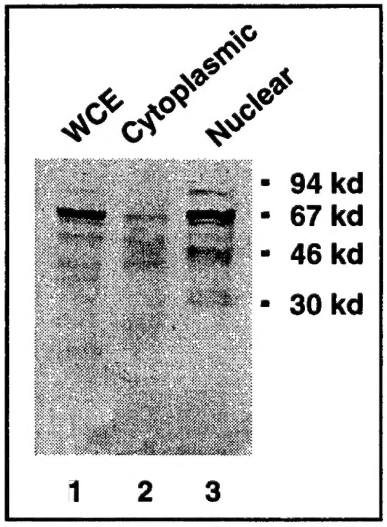

Fig. 1. Evidence for variant ER-α proteins in MCF-7 cells. Whole cell, cytoplasmic, or nuclear extracts from MCF-7 cells were resolved by SDS-PAGE, followed by western blotting using the ERα-specific monoclonal antibody, Mab-17.

at the RNA level (1-3), individual protein variants represent relatively minor species. However, since they are so numerous, in the aggregate variants constitute as much as 30-50% of ER-α immunoreactivity. Consistent with the observed subcellular localization behavior of wt ER-α and individual splicing variants (14), these ERα-related peptides partition in a characteristic pattern between the cytosol and the nucleus, with the 67 kd wt ER-α being primarily nuclear and several variants being primarily cytoplasmic (Fig. 1). While it is difficult to definitively rule out proteolysis as the cause for this size heterogeneity, data such as this supports the existence of stable protein variants of ER-α in estrogen-responsive tissues and cells.

To briefly summarize our findings on the functional activity of ER-α variants, all six of the variants analyzed (ERΔE2-ERΔE7, see Fig. 2) can be efficiently expressed in established cell lines such as Cos7, HeLa, and the ER-negative breast tumor line MDA MB231, ruling out speculation that ER-α splicing variants represent intrinsically unstable proteins. Each shows a characteristic pattern of subcellular distribution, with ERΔE3 and ERΔE5 being nuclear and the remaining variants (ERΔE2, ERΔE4, ERΔE6, & ERΔE7) displaying defective nuclear uptake (see Appendix A, Fig. 4). None of the variants binds efficiently to a consensus estrogen response element (ERE), although very weak DNA binding can be demonstrated for ERΔE5 and ERΔE7 (Appendix A, Fig. 2). Hormone binding by Scatchard analysis demonstrates that among the variants, only ERΔE3 binds 17β-estradiol (E_2) and does so with an affinity similar to wt ER-α (Appendix A, Fig. 3). Similarly, only ERΔE3 has the ability to form homologous dimers with itself or with wt ER-α (Appendix A, Fig. 6A). Both of these behaviors are consistent with the fact that only ERΔE3 retains an intact carboxy-terminal ligand-binding and dimerization domain (Fig. 2). As a consequence of its ability to dimerize, but its inability to bind to an ERE, ERΔE3 is capable of blocking DNA-binding by wt ER-α (17). Based on these findings, it is not surprising that none of the splicing variants are efficient inducers of transcription through a consensus ERE; however, ERΔE5 can display weak constitutive activity on ERE-containing reporters in some cellular contexts. Conversely, ERΔE3 and ERΔE5 reproducibly exert dominant inhibitory activity on an ERE-reporter when co-transfected with wt ER-α (Appendix A, Fig. 5 and ref. 18). The ability of both ERΔE3 and ERΔE5 to compete with wt ER-α for binding to p160 coactivators such as steroid receptor coactivator-1 (SRC-1) (Appendix A, Fig. 6B) is likely to explain, at least in part, the *inhibitory* activity of these variants on ERE-containing genes. Interaction with SRC-1 or other co-regulatory proteins is also likely to explain the ability of both ERΔE3 and ERΔE5 to exert *stimulatory* effects on gene expression that involve non-consensus estrogen response elements (described more fully below). The properties of these splicing variants are documented in the article appended to this report (Appendix A, ref. 14) and are summarized in Table I.

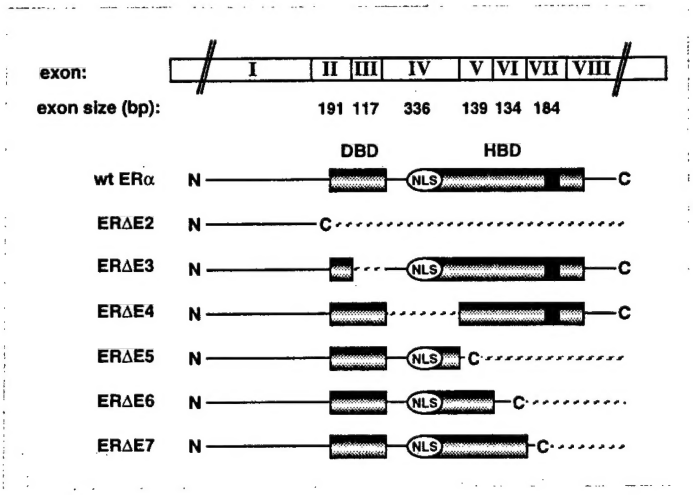

Fig. 2. Protein reading frames predicted from the sequences of cloned wt ER-α splicing variants, ERΔE2 through ERΔE7. Abreviations are N, amino terminus, C, carboxy terminus, NLS, nuclear localization signals, DBD, DNA-binding domain, and HBD, hormone-binding domain. The black rectangle at the end of the HBD represents the primary dimer contact site between ER-α subunits. Cross-hatched lines indicate portions of the ER-α reading frame that are not translated in each variant.

Table I. Biochemical Properties of ER-α Splicing variants								
Isoform	Size of reading frame (amino acids)	Predicted MW (kd)	Cellular Localization	DNA Binding Activity	Ligand Binding Activity	Subunit Dimerization	Transcriptional Activity	
							consensus ERE	non-consensus (AP1) sites
wt ERα	595	66.6	nuclear	intact	intact	intact	strong	variable
ERΔE2	152	17.0	cytoplasmic	n.d.	n.d.	n.d.	n.d.	n.d.
ERΔE3	556	62.3	nuclear	n.d.	intact	intact	n.d.	variable
ERΔE4	483	54.1	cytoplasmic	n.d.	n.d.	n.d.	n.d.	n.d.
ERΔE5	371	41.6	nuclear	weak	n.d.	n.d.	weak	variable
ERΔE6	473	53.0	cytoplasmic	n.d.	n.d.	n.d.	n.d.	n.d.
ERΔE7	466	52.2	cytoplasmic	very weak	n.d.	n.d.	n.d.	n.d.

* n.d., non-detectable.

Our emphasis during the final year of this project was on confirming preliminary observations that, indeed, several of the ER-α splicing variants possess gene-specific transcriptional stimulatory effects on promoters that contain non-consensus hormone response elements, but lack a consensus ERE. In recent years, a novel pathway for regulation of transcription by estrogen receptors (both ER-α and ER-β) has been described that involves co-operation of the receptors with the AP-1 transcription factors, c-jun and c-fos (19-22). This has been broadened to include other non-consensus EREs for which estrogen regulation has been mapped to transcription factor binding sites including SP1 (23-25) and a CRE (cyclic AMP Response Element)-like motif (26). An important feature of these non-classical pathways for ER action is that functional domains within the receptor that are crucial for transcriptional activation through an ERE are in some cases dispensable for ERα activity on AP-1 or SP 1-directed promoters. This led us to consider the possibility that the nuclear ERα splicing variants (namely ERΔE3 and ERΔE5) may function primarily in transcriptional regulation through non-consensus EREs. Four promoters were identified that are reproducibly stimulated by either ERΔE3 or ERΔE5, in addition (or in some cases in contrast) to wt ER-α.

Additional transfection experiments with the chicken ovalbumin promoter completed since the previous report confirmed that ERΔE3, like wt ER-α, can activate this gene. This response is a complex one in that it requires not only the presence of estradiol, but also activation of AP-1 by a phorbol ester (see Appendix A, Fig. 7). Phorbol 12-myristate, 13-acetate (PMA) serves to stimulate c-Jun N-Terminal Kinase (JNK) by directly activating protein kinase C, which in turn promotes increased phosphorylation of c-jun. That this effect is mediated by AP-1 is supported by our observation that induction of the ovalbumin reporter by both wt ER-α and ERΔE3 is enhanced by co-transfection with c-jun (Appendix A, Fig. 8). A requirement for direct binding of ER-α to this promoter is excluded by the fact that ERΔE3 is devoid of DNA-binding activity, arguing that activation of ovalbumin by these receptors is mediated indirectly through specific protein-protein interactions. However, repeated efforts to demonstrate a direct interaction between wt ER-α (or ERΔE3) and c-jun using a glutathione-S-transferase (GST) "pull-down" strategy have so far been unsuccessful.

Due to the stimulatory behavior of ERΔE3 on the ovalbumin promoter which contains an AP-1 site and half sites resembling an incomplete ERE (19), it was interested to examine the effects of the various ERα splicing variants on a reporter plasmid containing an AP-1 site in the context of a promoter not otherwise regulated by ER-α or estrogens. For this purpose, we chose p(AP1)$_3$-TK-CAT, which contains three consensus AP-1 sites upstream of the thymidine kinase promoter from *Herpes simplex* Virus. Somewhat unexpectedly, ERΔE5 (but not wt ER-α, ERΔE3, or any of the remaining splicing variants) was able to induce this promoter approximately seven fold in HeLa cells (Fig. 3). This response required activation of endogenous AP-1 by PMA, but was independent of estradiol (data not shown), consistent with the inability of ERΔE5 to bind ligand. While a widespread impression exists that ER-α is able to act as an estrogen-dependent inducer of transcription through a consensus AP-1 response element, the data presented here actually agree well with a published report showing no effect of wt ER-α and estradiol on an analogous AP-1/TK reporter construct (20). These data further suggest that the context of an AP-1 site within a promoter is critical for its ability to respond to wt ER-α or its splicing variants.

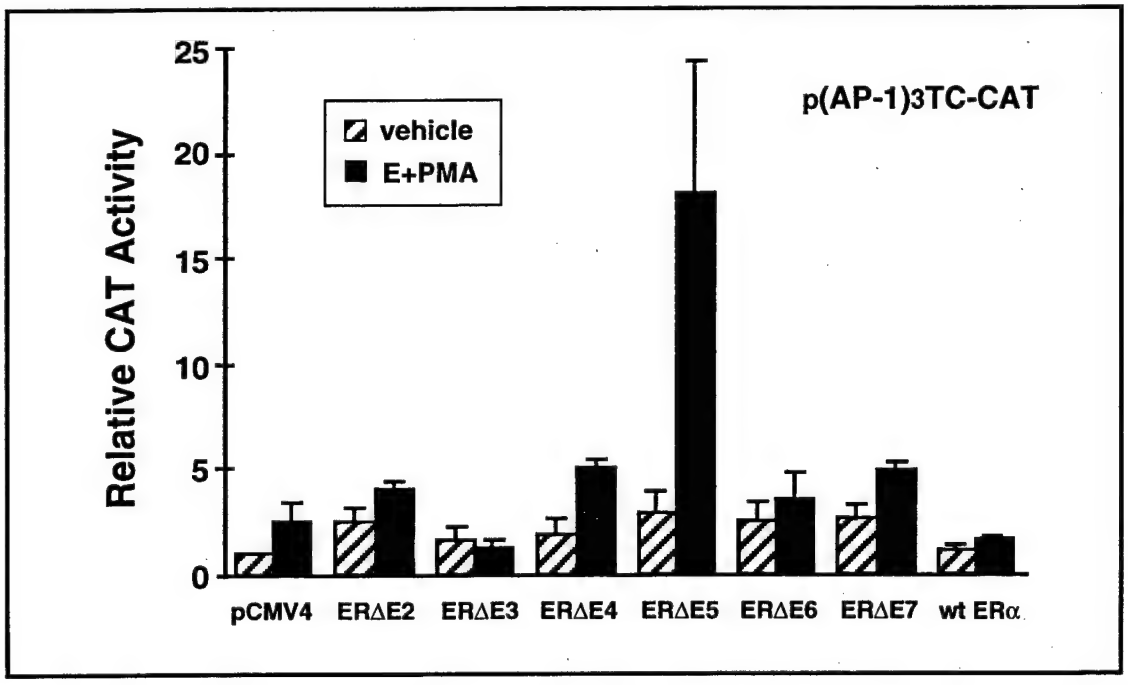

Fig. 3. Transcriptional stimulatory activity of ER-α splicing variants on a co-transfected p(AP1)$_3$TK-CAT reporter plasmid. HeLa cells were transiently transfected with pCMV vectors expressing each of the indicated receptor isoforms, followed by treatment for two days with vehicle (ethanol + DMSO, 1% each) or 10 nM 17β-estradiol + 20 nM PMA (E + PMA). Chloramphenicol acetylase (CAT) activities were normalized for protein and results are expressed relative to untreated empty expression vector (pCMV4) controls. Error bars represent the standard error of the mean of three independent experiments.

An additional promoter that has received considerable attention as a target for non-classical regulation by ER controls expression of the human collagenase gene. A short region of this promoter (-73 to +63 relative to the transcription start site) harbors an AP-1 element that is described to direct estrogen- or tamoxifen-regulated gene expression of a luciferase (Luc) reporter gene in a variety of cell lines (20-22). In contrast to these reports, we find that wt ERα behaves no differently on the Coll-73 Luc promoter than the empty pCMV4 control vector. In both cases, a modest (~2-fold) increase is observed that depends on activation of endogenous AP-1 by PMA treatment. The inclusion of either estradiol (10 nM) or tamoxifen (100 nM) together with PMA had no effect on the activity of Coll-73 Luc co-transfected with wt ERα (or pCMV4) compared to PMA alone (data not shown). However, this promoter can be dramatically activated by ERΔE3 and to a lesser extent by ERΔE5 in response to combined treatment with estrogen and PMA

(Fig. 4a). Like wt ER-α, the remaining splicing variants (ERΔE2, ERΔE4, ERΔE6 & ERΔE7) were without effect. Further analysis indicates that maximal activation of Coll-73 Luc by ERΔE3 requires co-treatment with both estradiol and PMA (but not tamoxifen and PMA) (Fig. 4b). Phorbol ester alone (or in combination with ER ligands) supports a modest, but statistically significant induction of the collagenase reporter that once again involves activation of endogenous AP-1 and is independent of co-expressed receptor. The human collagenase promoter therefore appears to represent a hybrid between the behaviors of $p(AP1)_3$-TK-CAT and pOvalb-CAT with respect to activation by ER-α splicing variants. Although estrogen regulation of all three of these promoters has been mapped to an AP-1 motif, differences in the relative strength of activation by the various receptor isoforms once again indicates that the context of the AP-1 site relative to other promoter elements may confer differences in the mechanism of AP-1 activation by ER-α, as well as the structural requirements for the ERα co-regulatory partner.

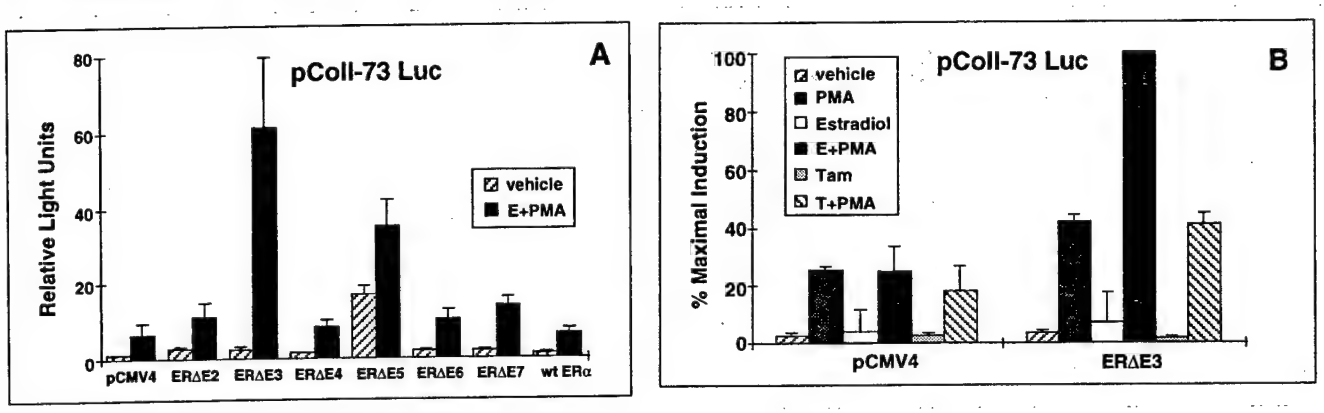

Fig. 4. Transcriptional stimulatory activity of ER-α splicing variants on a co-transfected human collagenase reporter plasmid, pColl-73 Luc. HeLa cells were transiently transfected as described in the legend to Fig. 3. Luciferase activities were normalized for protein and results are expressed relative to vehicle-treated empty expression vector (pCMV-4) controls. Error bars represent the standard error of the mean of three independent experiments. Panel (a) shows the behaviors of ER-α and individual ER-α splicing variants; panel (b) shows a breakdown of treatment conditions: 20 nM PMA with or without 10 nM 17β-estradiol or 100 nM tamoxifen (Tam) for pColl-73 Luc co-transfections with pCMV-4 or pCMV-ERΔE3.

The final promoter that we examined for activation by ER-α or its splicing variants is from the human Insulin-like Growth Factor-1 (IGF-1) gene. Expression of IGF-1 is stimulated by estradiol in the uterus of ovariectomized/hypophysectomized rats (27) and in cultured rat osteoblast cells (28). Complicating this picture are independent data showing that in primary fetal rat osteoblasts estradiol can block the increase in IGF-1 synthesis caused by parathyroid hormone or prostaglandin E_2 (29). These workers have further shown that this hormonal regulation of IGF-1 expression is mediated by a binding site for CCAAT / enhancer-binding protein-delta (C/EBP-δ) located between positions +202 and +209 within exon 1 of this gene (30). While an estrogen responsive AP-1 site has been described in the chicken IGF-1 gene (31), this site is not conserved within the IGF-1 promoters of mammals. The human IGF-1 promoter is thus qualitatively different from the examples described above in that it lacks any consensus binding sites for either AP-1 or SP1 that might represent likely targets for regulation by ER-α.

When co-transfected into HeLa cells with wt ER-α or its exon-skipped variants, a luciferase reporter plasmid containing 1.63 kb of sequence upstream from the human IGF-1 promoter (pIGF1(-1630)Luc) does not respond to wt ER-α. However, it is *constitutively* induced by ERΔE5 (Fig. 5). Activation of Protein Kinase C by treatment with PMA has no effect on the stimulatory activity of ERΔE5, further arguing against the involvement of AP-1 in this response. It is unclear where within the sequence of the IGF-1 promoter the ERΔE5 regulation is directed and efforts to map this unusual regulatory element continue. Analysis of a 5'-deletion series of the IGF-1 promoter has been largely uninformative, since promoter actively is progressively lost in mutants containing only 926, 591, or 233 bp of 5'-flanking

sequence, while modest but significant ERΔE5-responsiveness remains even in the shortest promoter construct (data not shown). Similarly, attempts to transfer ERΔE5-responsiveness onto a heterologous promoter have been unsuccessful, suggesting that this effect may require complex transcription factor interactions within the context of the intact IGF-1 promoter. In future attempts to localize the ERΔE5 response element, studies are planned to test the effect of ERΔE5 on short internal deletions or clustered point mutations of the IGF-1 promoter.

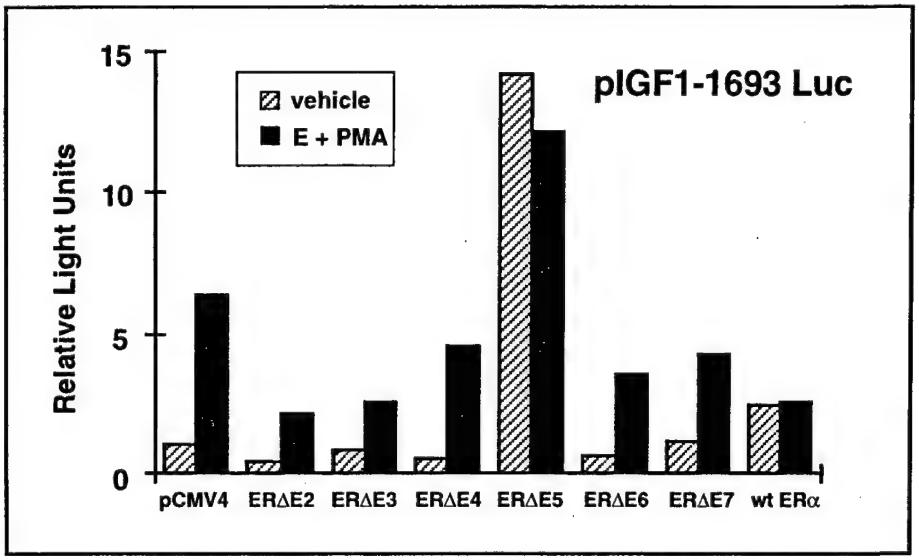

Fig. 5. Transcriptional activity of ER-α splicing variants on a co-transfected human IGF-1 reporter plasmid. HeLa cells were transiently transfected with pIGF1(-1630)Luc as described in the legend to Fig. 3. Luciferase activities were normalized for protein and results are expressed relative to vehicle-treated empty expression vector (pCMV-4) controls. Transfected cells were treated for 2 days with vehicle (V) or 10 nM 17β-estradiol plus 20 nM PMA (E + PMA). Error bars represent the standard error of the mean of three independent experiments.

A novel hinge region variant of ER-α results from the inclusion of a cryptic 39 bp exon encoded by a repetitive element within the third intron of the ER-α gene.

During the course of our analysis of ER-α splicing variants present in MCF-7 cells, we identified three cDNA clones that contained an identical insertion of 39 bp immediately downstream of exon 3. In one case, this novel insertion occurred precisely between exons 3 and 4 in an intact ER-α cDNA (clone 4F4), while in the remaining two cases (clones 2A2 and 6F1), it occurred at the junction of exon 3 in the context of further exon deletions (see Fig. 5 from the 3rd annual report). The net effect of this 39 bp addition is to cause the in-frame insertion of 13 new amino acids (Gly Asp Ser Gly Ile Ser Ser Trp Leu Gly Ser Ile Ala) just downstream of the DNA-binding domain of the ER-α peptide. This results in a 608 residue receptor with an expanded "hinge" region (Fig. 6). The molecular weight predicted for this variant (68.1 kd) would make it essentially indistinguishable from the 66.6 kd wt ER-α by any conventional protein analytical technique. A BLASTn search of GenBank indicates that this 39 bp element corresponds to a portion of a LINE 1 sequence, or Long Interspersed Element, Type PB1. LINE 1 elements are ancient, dispersed retrotransposons present at around 10^5 copies in all mammal genomes and constituting up to 30% of the mass of genomic DNA (32). Sequence comparisons indicate that the 39 bp motif present in these variant ER-α cDNAs is immediately flanked in the typical LINE 1 PB1 element by a consensus splice donor (GT) and acceptor (AG) signal, suggesting that it represents a cryptic "mini-exon" that can be alternatively spliced between exons 3 and 4 into the body of the ER-α mRNA (Fig. 6C). While the 3rd intron of the human ER-α gene has not yet been sequenced in its entirety, current evidence indicates that this intron exceeds 32 kb in size (31). Our data predicts that this intron must contain a LINE 1 PB1 repeat that occasionally contributes the 39 bp mini-exon to the processed ER-α transcript. Of interest, a clone

containing an unspecified 39 bp insertion between exons 3 and 4 was independently isolated by van't Veer and coworkers from MCF-7 cells, but was not further described (33).

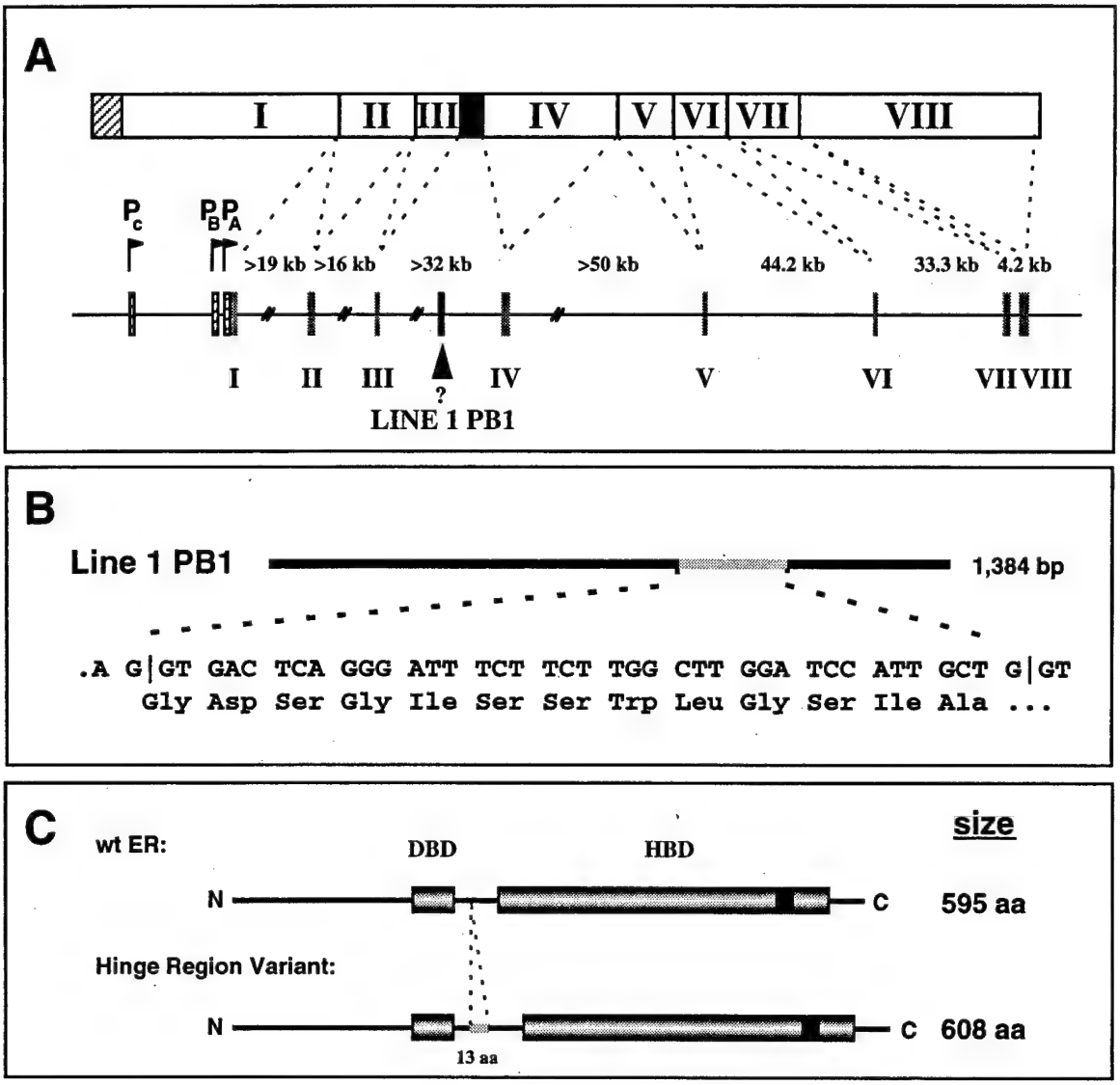

Fig. 6. A LINE 1 repetitive element within the ER-α gene contains a cryptic hinge region exon. Panel (a) depicts the organization of the ER-α gene and the predicted location of an L1 PB1 element ; panel (b) shows the sequence of the cryptic 39 bp mini-exon contained within the LINE 1 repeat; panel (C) indicates the position of the resulting 13 aa insertion within the hinge region of ER-α.

Previous studies of engineered mutants of ER-α that contain insertions between the DNA- and ligand-binding domains indicate that the hinge region of this receptor can tolerate insertions of at least 14 amino acids without noticeably compromising function (35). For this reason, it was of interest to assess the transcriptional activity of the 608 aa ER-α variant. To permit these experiments, a Not I / Hind III restriction fragment containing the 39 bp insertion was transferred into an otherwise wt ER-α cDNA within a pCMV4 expression vector. Fig. 7 shows the results from a series of experiments in which this 608 aa variant was co-transfected into HeLa cells together with consensus (pERE-TK-CAT) or non-consensus (pOvalb-CAT) reporter plasmids. For both reporters, the 608 aa variant behaved

indistinguishably from wt ER-α in its ability to stimulate transcription in response to estradiol (or estradiol + PMA). In experiments not shown, we have also determined that the 608 aa variant binds estradiol with normal affinity and localizes correctly to the nucleus. Unlike other ER-α splicing variants, the 608 aa receptor can also bind efficiently to an ERE in gel mobility shift assays (data not shown). Thus in every respect so far analyzed, the 608 aa ER-α variant appears to retain normal receptor function. We have not yet determined if the altered hinge region might exert subtle effects on DNA-binding affinity or specificity, thereby conferring on this receptor a unique target gene specificity. Other possibilities that merit further investigation concern changes in the prevalence of this variant in various tissues (e.g., breast, uterus, brain, bone, etc.) or physiological states (e.g., normal vs. tumor). This novel ER-α variant represents the subject of further study and our findings will be reported in the literature following completion of this work.

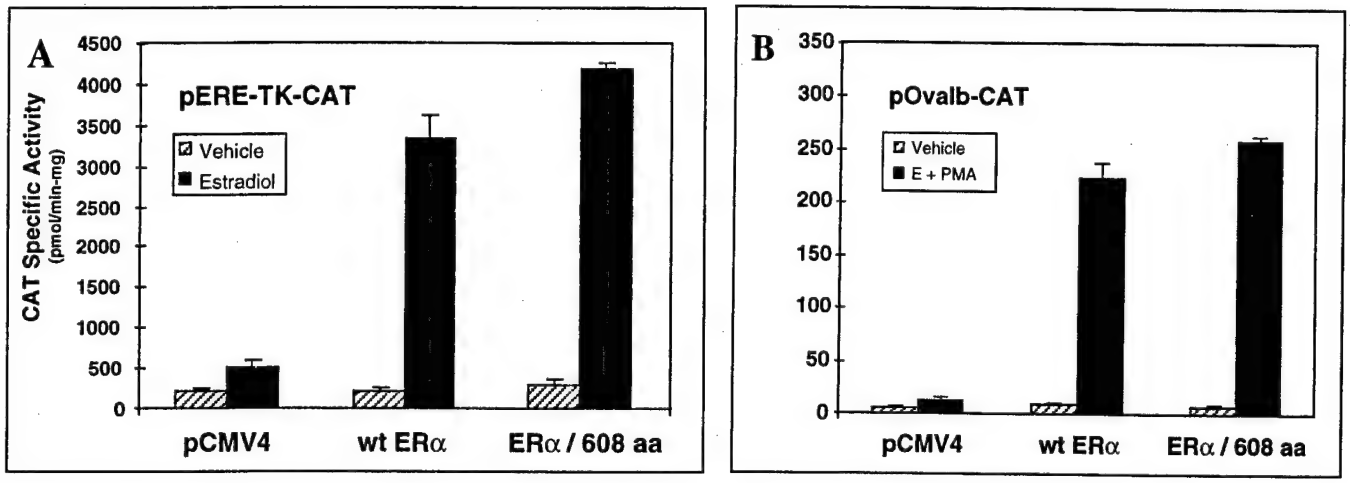

Fig. 7. Comparison of the transcriptional stimulatory activity of wt ER-α and the 608 aa Hinge Region Variant on consensus and non-consensus estrogen response elements. Panel (a) shows the behaviors of the ER-α and ER-α/ 608 aa receptors co-transfected with pERE-TK-CAT in the absence or presence of 10 nM 17β-estradiol compared with the empty vector, pCMV4; panel (b) shows their activities when co-transfected with pOValb-CAT, without or with 10 nM 17β-estradiol + 20 nM PMA. CAT activities were determined for reactions containing 100 ug of protein. Error bars represent the standard error of the mean for three independent experiments.

Characterization of cell lines that stably express the ERΔE5 variant:

As detailed in the annual report from year 3, a series of cell lines were established that stably express ERΔE5 in either a Cos7 or MDA MB231 cell background. Parallel efforts to stably express wt ER-α for control purposes were repeatedly unsuccessful. Consistent with our findings from the transient transfection experiments summarized above, stably expressed ERΔE5 was unable to bind to an ERE in mobility shift experiments and failed to stimulate the expression of a consensus ERE reporter (pERE-TK-CAT) introduced transiently into the stable cell lines. As a result of the difficulty of sustaining ERΔE5 expression in these cell lines through extended cell passages and our failure to observe any effects on their growth properties that could be attributed to expression of this ER-α variant, work with these cell lines was not further pursued. While this work was in progress, two excellent, but contradictory studies were published by independent laboratories on the consequences of stably expressing ERΔE5 in MCF-7 cells. In one case, Fuqua and colleagues reported that such cells display elevated PR expression, increased anchorage-independent colony formation in the absence of estrogen, and decreased sensitivity to the growth inhibitory effects of tamoxifen (5). However, in agreement with our observations Rea and Parker reported that ERΔE5 failed to alter the growth properties of MCF-7 cells, failed to induce expression of the endogenous PR or pS2 genes, and failed to confer tamoxifen resistance on these cells (36).

Task 2, Conclusions:

The major conclusions from the studies undertaken under Task 2 can be summarized as follows:

1) Exon-skipped ER-α mRNAs support the expression of stable, receptor-related peptides
2) Among the many ER-α splicing variants observed, the two with the greatest potential to modulate gene expression are ERΔE3 and ERΔE5, since their nuclear uptake is not impaired and since their conformations still permit them to interact with other transcriptional co-regulators
3) ERΔE3 and ERΔE5 interfere with the expression of genes containing consensus DNA-binding sites for ER-α, in part through direct inhibition of DNA-binding and in part through competition for limiting co-regulatory factors
4) ER-α splicing variants ERΔE3 and ERΔE5 display promoter-specific stimulatory effects through non-consensus estrogen regulatory elements
5) ERΔE3 and ERΔE5 therefore have the potential to exert estrogen regulatory effects or modulate the activity of ER-α in proportion to their relative abundance in ER-positive tissues and cells.

KEY RESEARCH ACCOMPLISHMENTS

- analysis of the profile of ER-α splicing variants in four breast epithelial cell lines (MCF-7, MCF10A TG-1, MCF10A TG-3, and M13 SV1)
- analysis of ER-α splicing variants in a breast tumor with discordant ER status
- identification of numerous novel ER-α splicing variants including a hinge region variant that incorporates a cryptic exon encoded by a LINE 1 repetitive element
- identification of ERΔE3 as a major ER-α splicing variant in the tamoxifen resistant cell line, MCF-7/LCC2
- development of an ERΔE5-specific monoclonal antibody
- characterization of the biochemical properties of the ER-α splicing variants ERΔE2, ERΔE3, ERΔE4, ERΔE5, ERΔE6, & ERΔE7
- analysis of the transcriptional activities of ER-α splicing variants, focusing on ERΔE3 & ERΔE5
- Identification of the non-classical (ERE-independent) pathway for estrogen regulation as the major means for transcriptional regulation by ERΔE3 & ERΔE

DISCUSSION

As we and others have repeatedly shown (1-3), the ER-α gene gives rise to a markedly heterogeneous population of RNA transcripts that includes exon-skipped, as well as correctly spliced ER-α mRNAs. Splicing variants of ER-α are present in all estrogen-responsive tissues, including both the normal and cancerous breast. They are also readily detected in breast epithelial cell lines such as MCF-7 and MCF10AT. This project had two primary goals: 1) to better define the full spectrum of variant ER-α mRNAs that exist and 2) to more fully characterize the biochemical properties and transcriptional activities retained by the most prevalent of these ER-α variants. Results from this project call further into question the appealing, but simplistic notion that all of the biologically important regulatory effects of ER-α are mediated by a single 595 aa form of this receptor that was originally described by Chambon and colleagues in 1986 (37). Rather, there is growing evidence that ER-α splicing variants (most notably ERΔE3 and ERΔE5) have a significant impact on the ability of the breast and other tissues to respond to estrogens.

Structural analysis of ER-α cDNAs by PCR amplification, cloning, and sequencing from a variety of breast epithelial cell lines revealed a total of 54 discrete ER-α variants, most of which are the result of the precise deletion of one or more of the six internal exons of this gene (i.e., exons 2-7). This also includes a number of less abundant, but more diverse clones that harbor internal deletions with endpoints that do not correspond to known splice donor or acceptor sites. In many cases, these appear to be the product of cryptic splicing signals or to result from promiscuous splicing at non-consensus donor or acceptor sites. A variety of studies from independent laboratories indicate that ERΔE7 represents the most abundant of the variants (accounting for 10-15% of ER-α mRNA). Other variants, such as ERΔE3, ERΔE4, and ERΔE5 each represent approximately 1-5% of the ER-α mRNA pool. Together, however, ER-α splicing variants can make up 50% or more of ER-α mRNA giving credence to suggestions that they are a significant factor in the estrogen signal transduction pathway. Studies investigating changes in the ratio of correctly spliced to variant transcripts remain inconclusive, although there have been several suggestions that ERΔE5 and ERΔE7 tend to be elevated relative to ER-α mRNA in breast tumors (38) or in a subset of PR- and pS2-positive tumors that were scored as ER-negative by immunohistochemical analysis (39). The opposite behavior was more recently reported for ERΔE3, which was found to be *reduced* in breast tumors relative to normal tissue (40). Based on functional analysis of ERΔE3, these authors further suggest that re-expression of this variant at levels more characteristic of normal tissue *suppresses*, rather than enhances the transformed phenotype of stably transfected MCF-7 cells. Interestingly, we observed a similar pattern of lower ERΔE3 expression in the MCF-7 tumor cell line compared with non-tumorigenic MCF10AT cells. This finding is consistent with observation that ERΔE3 can block the ability of ER-α to stimulate ERE-containing genes that are thought to be involved in proliferative pathways, while independently using the non-classical (ERE-independent) pathway of estrogen action to stimulate genes that may be involved in differentiated functions of breast tissue.

Our analysis of splicing variants indicates that, while all of the variants can be stably expressed, ERΔE2, ERΔE4, ERΔE6, and ERΔE7 represent functionally impaired receptor isoforms that fail to localize correctly to the nucleus and have no demonstrable effects (either stimulatory or inhibitory) on gene expression (15). Whether these variants fulfill some as yet undefined role within the cytoplasmic compartment is currently unknown. It should be stressed, however, that none of the cytoplasmic ER-α variants can bind ligand. The ERΔE3 and ERΔE5 variants, in contrast, represent nuclear isoforms that retain at least some aspects of receptor function. For ERΔE3, this includes the ability to bind ligand, to form homologous or mixed dimers with itself or wt ER-α, respectively, and to interact with at least some of the same co-regulatory proteins that enable wt ER-α to function. Although it is unable to bind ligand or dimerize, the ERΔE5 variant can bind weakly to DNA and also interacts with nuclear receptor co-activators, albeit not as efficiently as either ERΔE3 or wt ER-α. These properties enable both ERΔE3 and ERΔE5 to *interfere* with the stimulatory activity of wt ER-α on ERE-containing genes and, more importantly, to *mimic* the activity of wt ER-α in ERE-independent gene regulatory pathways. It is therefore reasonable to propose that over-expression of the nuclear splicing variants ERΔE3 and ERΔE5 relative to wt ER-α has the potential to shift the pattern of gene expression away from ERE-containing genes to those that are regulated non-classically by estrogens.

To further examine this possibility in a model system, we have tested the effect of co-transfecting individual ER-α splicing variants, compared with an empty expression vector, on the activity of four reporter plasmids that were selected from the literature based on their ability to be transcriptionally induced by ER-α, despite their apparent lack of canonical EREs. These plasmids included pOvalb-CAT (driven by a 1350 bp promoter fragment from the chicken ovalbumin gene), $p(AP1)_3$TK-CAT (driven by three consensus AP-1 sites upstream of the HSV thymidine kinase promoter), pColl(-73)Luc (driven by the -73/+63 human collagenase promoter), and pIGF(-1630)Luc (driven by the -1630/+322 human IGF-1 promoter). The actual behavior of these reporters in response to the various receptor isoforms was not readily predicted from their known biochemical properties and significant differences are evident for each of the reporters tested (see Table II). In contrast to the reporter plasmids shown, neither wt ER-α, ERΔE3, or ERΔE5 had any significant effect on a variety of control plasmids including promoters for SV40 T-antigen, c-Met, or the thymidine kinase promoter by itself. Of note, three of the reporter plasmids lacking a consensus ERE (i.e., pColl(-73)Luc, $p(AP1)_3$TK-CAT, and pIGF(-1630)Luc) were stimulated by one or both of the splicing variants, but showed no significant response to wt ER-α. In several cases (notably pOvalb-CAT and pColl(-73)Luc) the regulatory behavior of these genes displayed an added complexity, requiring both estradiol and phorbol ester for maximal activity. On the other hand, $p(AP1)_3$TK-CAT and pIGF(-1630)Luc which were stimulated only by ERΔE5 showed no estrogen responsiveness, consistent with the inability of this variant to bind ligand.

Table II: Activity of wt ER-α, ERΔE3, and ERΔE5 on Various Promoters

reporter	wt ER-α	ERΔE3	ERΔE5
pERE-TK-CAT	Induced by Estradiol	No Effect	Very Weakly Constitutive
pOvalb-CAT	Induced by Estradiol + PMA	Induced by Estradiol + PMA	Weakly Constitutive
pColl(-73)Luc	No Effect	Induced by Estradiol + PMA	Modestly Induced by PMA
$p(AP1)_3$TK-CAT	No Effect	No Effect	Induced by PMA
pIGF(-1630)Luc	No Effect	No Effect	Constitutive

Mechanistically, we believe that this phenomenon involves protein-protein interactions between ER-α and jun, fos, SP1, or other upstream transcription factors acting through their cognate DNA-response elements. Among the genes examined, the ovalbumin and collagenase promoters (as well as the $p(AP1)_3$TK-CAT reporter) contain at least one consensus phorbol ester response element (also known as a TRE or an AP-1 site). While estrogen regulation of the chicken ovalbumin promoter was originally believed to involve cooperative binding of ER-α to low affinity sites resembling portions of a palindromic ERE, these "half-sites" have been reinterpreted to represent an AP-1 motif. Indeed, induction of this promoter was clearly shown not to require the direct binding of ER-α to DNA (19). Consistent with this fact, we observe that ERΔE3 which also fails to bind to DNA is nonetheless active on this reporter plasmid. Even though ERΔE5 may possess very low affinity for a consensus ERE, binding of ERΔE5 to DNA is unlikely to occur under physiologic conditions. Certainly, the ability of ERΔE5 to stimulate the collagenase and IGF-1 promoters does not involve DNA-binding, as these promoters also lack consensus ERE motifs. Differences we observe in the behavior of these promoters also indicate that the context of the AP-1 motif (or other non-canonical ERE) can exert a significant effect on how these promoters respond to ER-α and its variants. Since only some of these

differences can be ascribed to structural differences between the variants, differences in the identity of the transcription factors or co-regulators involved in the expression of each of these genes must also be involved.

As noted above, stimulation of transcription by ERΔE3 invariably requires dual activation by both estradiol and phorbol ester, in clear contrast to wt ER-α which can induce ERE-containing genes in response to estradiol alone. The situation for ERΔE5 is less clear, since efficient induction of the collagenase and AP1 reporters by this splicing variant requires simultaneous treatment with phorbol ester, while stimulation of the IGF-1 promoter does not. We interpret these findings to implying that gene activation by ERΔE3 (and in some, but not all cases by ERΔE5) involves the recruitment of transcriptional regulatory proteins through their intrinsic activation domains. Two activation functions have been described in wt ER-α, one within the amino-terminus (commonly referred to as AF1) that is shared by both ERΔE3 and ERΔE5. A second estrogen-inducible domain (known as AF2) resides within the carboxy-terminal ligand binding domain and is therefore present in ERΔE3, but not in ERΔE5. Studies performed using wt ER-α, demonstrate that these activation functions correspond to "docking sites" for various co-regulatory proteins including CBP/p300 or p160 co-activators such as SRC-1, TIF-2, GRIP1, or AIB-1. Indeed, we can demonstrate that both ERΔE3 and ERΔE5, like wt ER-α, can interact with the nuclear receptor coactivator SRC-1 (15). For ERΔE3, this interaction requires at least two SRC-1 binding sites and is dramatically stimulated by ligand. Only the constitutive amino-terminal site (AF1) is present in ERΔE5. Whether transcriptional stimulation of reporters containing non-canonical hormone response elements (such as pOvalb-CAT or pColl(-73)Luc) involves a direct interaction between ER-α or its isoforms and the AP-1 components jun and fos, or an indirect interaction mediated through a bridging factor such as CBP/p300 or SRC-1 still needs to be clarified. Additionally, the identity of the downstream target(s) for activation by phorbol ester, while presumed to involve the protein kinase C pathway, remain to be clarified. Targets that must be considered include AP-1, the nuclear receptor co-regulators, or the ER-α isoforms themselves. A general model comparing ER-α action through consensus and non-consensus hormone response elements is presented in Fig. 8.

While our transfection studies fail to directly address the impact that ERΔE3 and ERΔE5 have on the transcription of endogenous genes in breast tissue or in tumors, these results suggest that they nonetheless have the potential to make distinctive and possibly unique contributions to patterns of gene regulation. As an extracellular matrix protease, collagenase is likely to play an important role in the tissue remodeling and stromal invasion that occurs in metastatic breast disease. The IGF-1 gene is similarly expressed in a variety of tissues, including the liver, bone, uterus and breast, where it contributes to the normal growth and differentiation of these structures (41). Some uncertainty remains regarding the sources of IGF-1 within the breast and with respect to its precise role in breast tumor growth. It is likely that the mammary epithelium is exposed to IGF-1 produced by the breast stroma as well as from systemic sources such as the liver. In addition, some breast tumors produce IGF-1 and other growth factors of their own (42). Expression of IGF-1 (and other growth factors) appears to be deregulated in some tumors, contributing an autocrine component to tumor cell growth. The ability of ERΔE5 to stimulate transcription of the IGF-1 gene in breast tumor cells is therefore of potential clinical significance. Interestingly, we observe that ERΔE5, but not wt ER-α promotes expression of a co-transfected IGF-1 reporter plasmid,

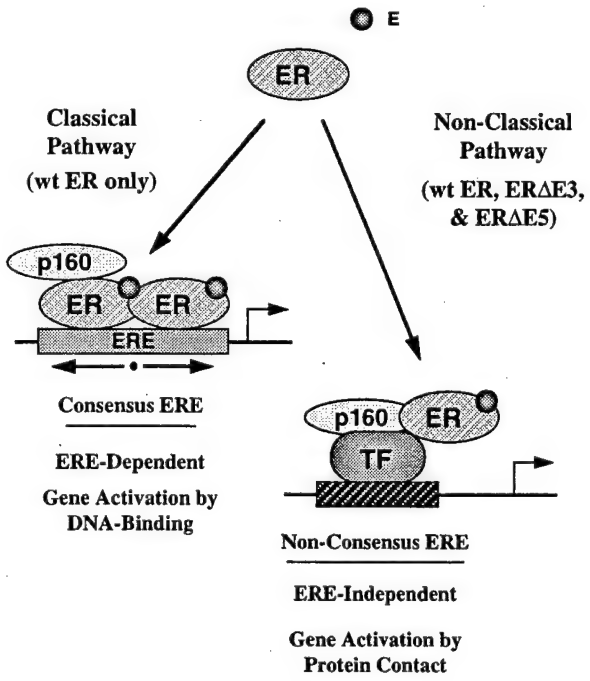

Fig. 8. Model for the mechanism of transcriptional activation by ER-α and its splicing variants through consensus and non-consensus estrogen regulatory elements.

predicting that IGF-1 production by breast tumors will be insensitive to tamoxifen. Experiments are in progress to map the cis element within the IGF-1 promoter that is responsible for this regulation and to explore the implications of this finding.

Our finding that the ERΔE3 and ERΔE5 splicing variants can exert stimulatory effects on gene expression through non-canonical estrogen regulatory elements may have broader implications, as a growing number of estrogen-responsive genes have been reported to lack a consensus DNA-binding site for ER-α. Among these are genes such as cathepsin D, cyclin D1, c-fos, c-myc, and RARα that are believed to play important roles both during normal breast development and during tumor formation or tumor progression. Notably, the structural requirements for ER-α to regulate many of these non-canonical hormone response elements are less stringent than transcriptional stimulation through a consensus ERE, suggesting that ERΔE3 and ERΔE5 may have significant effects on these genes as well. The traditional view of wt ER-α as the central player in target gene selection by virtue of its ability to bind to the ERE motif may therefore need to be modified to include estrogen regulation through the non-classical AP-1 and SP1 pathways and to accommodate the participation of the ERΔE3 and ERΔE5 splicing variants. Our observations provide the first solid evidence that selected splicing variants, like wt ER-α itself, can exert significant positive effects on gene transcription and raises the intriguing possibility that each one of these receptor isoforms may target a unique, but overlapping subset of genes for transcriptional control.

REPORTABLE OUTCOMES

Publications:

1) Publications from this award:

a) From most recent award year (copy included as Appendix A):

- Bollig, A. and Miksicek, R.J. (2000) An estrogen receptor-α splicing variant mediates both positive and negative effects on gene transcription. *Molecular Endocrinology*, 14(5):634-649.

b) Previous publications related to this award:

- Bunone, G., Briand, P.-A., Miksicek, R.J., and Picard, D. (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J.*, 15(9):2174-2183.
- Elias, J.M., Hyder, D.M., Miksicek, R.J., Heimann, A., and Margiotta, M. (1995) Interpretation of steroid receptors in breast cancer: a case with discordant estrogen receptor results using ER1D5 and H222. *J. Histotechnology*, 18(4):331-335.

c) Previous abstracts from this award:

- Bollig, A.B., Ankrapp, D.P., and Miksicek, R.J., Functional analysis of estrogen receptor splicing variants. 79Th Annual Meeting of the Endocrine Society, Minneapolis, MN (June, 1997), Abstract P3-420.

d) Manuscripts still in preparation from this award:

- Ankrapp, D.P., Morrison, M., Bollig, A.B., and Miksicek, R.J., MCF-7 and MCF10AT breast epithelial cells express a broad array of estrogen receptor-α mRNA size variants, in preparation.
- Bollig, A.B. and Miksicek, R.J., The estrogen receptor-α splicing variants ERΔE3 and ERΔE5 display promoter-specific effects through non-consensus estrogen regulatory elements, in preparation.
- Ankrapp, D.P., Morrison, M., Bollig, A.B., and Miksicek, R.J., A conditional exon encoded by a Line 1 element within the third intron of the estrogen receptor-α gene gives rise to a functional hinge region receptor variant, in preparation.

2) Current publications and manuscripts unrelated to this award:

- Wang S.F., Li C., Zhu J., Miura K., Miksicek R.J., and Raikhel A.S. (2000) Differential expression and regulation by 20-hydroxyecdysone of mosquito ultraspiracle isoforms. *Developmental Biology* 218(1):99-113.
- Miksicek, R.J., Lee, C., and Morrison, M., Synthetic peptides derived from dimerization motifs within estrogen receptor-α interfere with receptor DNA-binding, in preparation.

Portions of this work were presented at the following seminars and research presentations:

MSU Dept. of Surgery (May, 1997)
MSU Cancer Center (April, 1998)
MSU Cell and Molecular Biology Program, Fall Retreat (August, 1998)
MSU Breast Cancer Research Group, (November, 1999)
MSU Dept. of Physiology Research Forum (February, 2000)

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An Estrogen Receptor-α Splicing Variant Mediates Both Positive and Negative Effects on Gene Transcription

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Analysis of mRNA prepared from a variety of estrogen-responsive cell lines, breast tumor specimens, and normal breast tissue have established that estrogen receptor-α (ERα) mRNA is typically expressed as a mixture of transcripts. Using PCR amplification, this heterogeneity has been shown to result largely from an imprecise pattern of mRNA splicing that gives rise to a family of correctly processed and exon-skipped ERα transcripts. We have reconstructed ERα cDNAs representing the single exon-skipped variants ERΔE2 through ERΔE7 to enable their functional characterization in a well defined cell transfection system. All six of the ERα splicing variants support the efficient expression of stable proteins in Cos7 cells, and each shows a characteristic pattern of subcellular distribution. Each of the variants displays a dramatic reduction in DNA-binding activity with a consensus estrogen response element (ERE) in an *in vitro* gel mobility shift assay. While this DNA-binding defect appears to be complete for ERΔE2, ERΔE3, ERΔE4, and ERΔE6, weak DNA binding is observed for ERΔE5 and ERΔE7. Scatchard analysis of hormone binding demonstrates that among the variants, only ERΔE3 binds 17β-estradiol (E_2) and does so with an affinity similar to wild-type ERα (wt ERα). Individual variants cotransfected with the pERE-TK-CAT reporter plasmid [a consensus ERE-driven chloramphenicol acetyltransferase (CAT) reporter gene that is highly responsive to E_2-liganded wt ERα] were ineffective at inducing CAT expression in ER-negative HeLa cells. Only ERΔE5 showed indications of positive transcriptional activity on the pERE-TK-CAT reporter, but this activity was limited to approximately 5% of the activity of wt ERα. When variants were expressed simultaneously with wt ERα, ERΔE3 and ERΔE5 were observed to have a dominant negative effect on wt ERα transcriptional activity. Like the wild-type receptor, both ERΔE3 and ERΔE5 interact with steroid receptor coactivator-1e (SRC-1e) *in vitro*; however, only ERΔE3 retained the ability to dimerize with wt ERα. Transcription from a region of the ovalbumin promoter, which contains an ERE half-site and an AP-1 motif, is positively regulated by liganded wt ERα and ERΔE3 in phorbol ester-treated, transiently transfected HeLa cells. In both cases, this activity was enhanced by cotransfected cJun. These observations suggest that selected ERα splicing variants are likely to exert important transcriptional effects, especially on genes that are regulated by nonconsensus EREs and subject to complex hormonal control. (Molecular Endocrinology 14: 634–649, 2000)

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Molecular Endocrinology 14(5): 634–649

INTRODUCTION

Binding of estrogen to the estrogen receptor (ER) elicits a change in receptor conformation that allows the receptor to bind DNA and enhance transcription from the promoters of regulated genes (1–3). ER-induced gene expression supports the proliferation and, ultimately, the differentiation of target cells (4, 5). Interference with these proliferative effects forms the basis for the chemotherapeutic actions of estrogen antagonists that are used to treat cancers of the breast and reproductive tract (6). The reported success of antiestrogens such as tamoxifen and raloxifene in preventing breast tumors emphasizes a crucial role for ER in mammary carcinogenesis (7–9).

The transcriptional effects of estrogens are mediated by two closely related receptor isoforms, ERα and the more recently described ERβ (10, 11), each of which is encoded by a separate gene. While ERβ is also being investigated for its potential role in various diseases, including cancer, this study focuses solely on the ERα isoform. Analysis of mRNA prepared from a variety of estrogen-responsive cells and tissues, including breast tumors, has established that ERα mRNA is typically expressed as a mixture of transcripts (12–15). This heterogeneity results largely from a pattern of alternative mRNA splicing that gives rise to a family of correctly pro-

cessed and exon-skipped ERα mRNAs. ERα mRNA comprises sequences from 8 coding exons and is translated to yield a protein with discrete functional domains. An N-terminal transactivation function (AF1) encoded by exon 1 and a portion of exon 2 is thought to promote gene transcription by interacting with nuclear receptor coactivators and also with proteins integral to the transcription initiation complex (1, 16, 17). Derived from exons 2 and 3 is a centrally located zinc-finger motif (commonly referred to as the DNA-binding domain or DBD) that is essential for sequence-specific DNA binding and transcriptional activation through canonical estrogen response elements (EREs) (18). Within the region encoded by exon 4 are the nuclear localization signals (NLS) and a hinge region that allows for receptor conformational flexibility (3, 19). A ligand-binding domain (LBD) confers regulatory function to the receptor and is encoded by the C-terminal exons 4 through 8 (20). This region also includes determinants for subunit dimerization and a well characterized C-terminal transactivation function (AF2), which promotes gene transcription by recruiting coactivators (1–3, 21). Like other nuclear receptors, ERα is a modular protein in that individual domains are capable of demonstrating autonomous function within receptor mutants, as well as when they are introduced into heterologous fusion proteins (1, 18). It can reasonably be assumed that the exclusion of a particular exon will predictably result in a protein lacking the function ascribed to that exon. Additionally, it is probable that the loss of a particular exon will result in unpredictable functional deficits or perhaps even bestow a novel function on the variant receptor. This study examines the function of ERα splicing variants from the vantage point of what is known about the functional organization of wt ERα. Concurrently, the process of examining splicing variants, like mutational studies, improves our understanding of wt ERα function. We report results from experiments designed to assess receptor capacity to translocate to the nucleus, bind to DNA, bind ligand, participate in protein complexes, and promote gene transcription.

Fuqua and colleagues (22, 23) have reported that ERΔE5 (which contains the AF1 domain, but lacks AF2 and the regulatory functions imparted by the LBD), is constitutively active in promoting transcription from an ERE in a heterologous yeast reporter gene assay. These authors have also described that overexpression of ERΔE5 in a stably transfected breast cancer cell line (MCF-7) supported greater proliferation compared with control cells, as well as imparting a tamoxifen-resistant phenotype (24). In the human osteosarcoma cell line U2-OS, it has recently been reported that coexpression of ERΔE5 significantly enhances ERE-directed reporter gene expression induced by wt ERα (25). The existence of a constitutively active receptor variant (such as ERΔE5) able to exert a mitogenic effect in breast tumor cells in the absence of E_2 or in the presence of tamoxifen is an appealing explanation for the acquisition of antiestrogen resistance observed in previously responsive tumors and cell lines (26, 27). However, this model is challenged by conflicting observations that ERΔE5 and closely related, genetically engineered ERα mutants do not efficiently induce transcription from an ERE reporter in transiently transfected ER-negative HeLa or CEF cells (2, 28), or promote proliferation in stably transfected MCF-7 cells (28).

Recently, a novel mechanism for mediation of an estrogen response has been reported to involve AP-1-directed regulation of transcription by ER (29–32). AP-1 describes the fos/jun family of transcription factors that play a key role in transducing the effects of growth factors to regulate cell proliferation (33, 34). A variety of estrogen-responsive genes have been described that lack a palindromic ERE, but instead contain one or more consensus AP-1 elements (5′-TGAG/CTCA-3′), with or without a degenerate ERE or ERE half-site (5′-GGTCA-3′ or 5′-TGACC-3′). Examples of such genes include ovalbumin, which is induced by E_2 in chicken oviduct cells (35), and the insulin-like growth factor-I (IGF-I) gene whose expression is stimulated by E_2 in the uterus of ovariectomized-hypophysectomized rats and in cultured rat osteoblast cells (36, 37). An AP-1 enhancer motif identified in the chicken IGF-I promoter is essential for E_2 and phorbol ester-stimulated gene transcription (31). Phorbol esters act directly on protein kinase C to initiate a signal transduction cascade that ultimately activates AP-1 (33). Reporter gene cotransfection studies with expression vectors for AP-1 isoforms and ERα in HeLa cells indicate that a similar mechanism regulates the human collagenase promoter (32). The minimal region of the collagenase promoter reported to be responsive to tamoxifen-liganded wt ERα, and to a variety of ERα mutants, harbors a critical AP-1 element and lacks a consensus ERE. Additionally, the activity of ERα on the collagenase promoter was enhanced with AP-1 (c-*jun* or c-*fos*) overexpression (32). Further evidence that ER regulation converges with AP-1-directed gene transcription is provided by results from protein binding assays indicating that c-*jun* is able to bind to wt ERα *in vitro* (32).

Although evidence for function of ERα variants has been elusive, reports that ERΔE5 can support weak, cell type-dependent activity (23, 25, 28), and that, when tested on an ERE, both ERΔE5 and ERΔE3 are dominant negative receptor forms in the presence of wt ERα (38, 39) indicate that it is inaccurate to label these variants as transcriptionally inert. To investigate the capacity for ERα splicing variants to regulate gene transcription, we have expanded our transcriptional focus to include the noncanonical ERE of the ovalbumin promoter in addition to the consensus vitellogenin A2 ERE. Here we present data indicating that individual variants display both similarities and differences compared with wt ERα, and that selected splicing variants (specifically ERΔE3 and ERΔE5) have the ca-

pacity to both positively and negatively regulate gene expression, depending on the promoter context.

RESULTS

Numerous variant ERα cDNAs have now been cloned and sequenced from breast tumors and established tumor cell lines (12–15). The most common variants harbor a precise deletion of one of the internal exons from the eight that contribute to the structure of the mature ERα protein, suggesting that they arise as a result of imprecise splicing of the primary ERα mRNA transcript. ERα cDNAs with sequence deletions corresponding to exons 2, 3, 4, 5, and 7 have been identified, along with a large number of more complex variants (12–15). These basic variants will be referred to as ERΔE2 through ERΔE7, where the deleted exon is indicated numerically. Although there is no consistent ratio of relative expression, wild-type and variant ER transcripts are always coexpressed in ER- positive tumor cell lines and normal and tumorous breast tissue. Quantitation of individual variants shows that they generally represent a minority of ER mRNA; however, as a population, splicing variants typically constitute as much as 50% of the total ER mRNA in the tissues and cell lines examined (Refs. 12, 13, and 15 and D. P. Ankrapp and R. J. Miksicek, unpublished observations). While there has been extensive analysis at the RNA level of the pattern of expression and abundance of ERα splicing variants, limited information is available on their functional activity. We have therefore constructed cytomegalovirus (CMV) promoter-driven ERα cDNA expression vectors representing the exon-skipped variants ERΔE2 through ERΔE7 to enable their functional characterization in a well defined cell transfection system. Our assembled pool of ERα splicing variants also includes the hypothetical receptor ERΔE6, even though this variant is not readily identified *in vivo*. Figure 1A diagrams the ERα mRNA splicing variants examined, showing the positions of deleted exons and their consequences with respect to protein structure. Deletion of exon 2, 5, 6, or 7 all cause a frame-shift mutation resulting in premature termination of translation, thereby generating a diverse class of C-terminally truncated receptor forms. Omission of either exon 3 or 4 does not disrupt the mRNA reading frame, but produces a receptor protein with an internal deletion.

Transient expression in Cos7 cells demonstrates that each of these variants translates to a stable protein able to accumulate to readily detectable levels within transfected cells (Fig. 1B). Based on immunoblot analysis with an N-terminal monoclonal antibody (Mab-17), which recognizes an epitope within exon 1 common to all of the variants (40), we observe that the mobility of the six variant proteins is consistent with their predicted molecular weights. No immunoreactivty is observed in mock transfected cells, confirming the specificity of the Mab-17 antibody.

Measurement of the DNA-Binding Activity of the ERα Splicing Variants

Efficient DNA binding by ERα requires the cooperation of several functional elements within this protein, including the centrally located DBD and a ligand-inducible subunit dimerization motif located near the C-terminal end of the LBD (2, 18). It is also possible that additional subunit contacts occur elsewhere in the protein. Because all of the ERα splicing variants sustained deletions within various regions of this protein, it was of interest to systematically assess the DNA-binding ability of each variant. For this purpose, gel mobility shift assays were performed using extracts prepared from E_2-treated, transiently transfected Cos7 cells. Extracts were incubated with a ^{32}P-labeled oligonucleotide containing a consensus ERE (AGGTCACAGTGACCT) from the *Xenopus* vitellogenin A2 promoter. As expected, variants that harbor a mutation within the DBD (ERΔE2 and ERΔE3) are completely unable to recognize the ERE (Fig. 2, lanes 5–8). Less predictably, the loss of exons contributing to the LBD also result in a strong defect in ERE recognition (Fig. 2, lanes 9–16). For ERΔE5 and ERΔE7, however, this appears to be a quantitative defect in DNA binding. The addition of the monoclonal antibody, MAb-17, to the binding reactions consistently results in the recovery of weak DNA binding by ERΔE5 (Fig. 2, lane 12). Presumably, the role of the bivalent antibody is to stabilize the interaction of receptor subunits with their palindromic binding site, mimicking the function of the missing dimerization motif present within the C terminus of the LBD. These results suggest the possible existence of cell-specific constituents that perform the same function *in vivo* and may account for the variable activity of ERΔE5 and related constructs in different cell types (2, 25, 28). We have also observed the formation of a complex between ERΔE7 and labeled ERE probe (Fig. 2, lane 16, and data not shown). Overall, however, the relative weakness of DNA-binding observed in these studies raises serious questions about the extent to which any of the variants, including ERΔE5 and ERΔE7, are able to recognize and bind to a consensus ERE, *in vivo*. Furthermore, that ERΔE7 binds an ERE *in vitro* has little transcriptional relevance in light of the observation that ERΔE7 is not translocated to the nucleus when expressed in Cos 7 cells (see below).

ERΔE3, Like wt ERα, Binds Ligand

To test the ability of the ERα mRNA splicing variants to bind hormone, we performed a saturation binding assay on whole-cell extracts from Cos7 cells transiently transfected with wt ERα or the ERα variants. Only wt ERα and ERΔE3 were able to bind ^{3}H-labeled E_2, whereas all of the remaining variants demonstrated no specific ligand binding (Fig. 3A). The individual deletion of exons 2, 4, 5, 6, and 7 effectively eliminates all, or a significant portion, of the LBD (see Fig. 1A), consistent with their loss of hormone binding. We confirmed these results using an *in vivo* ligand-binding

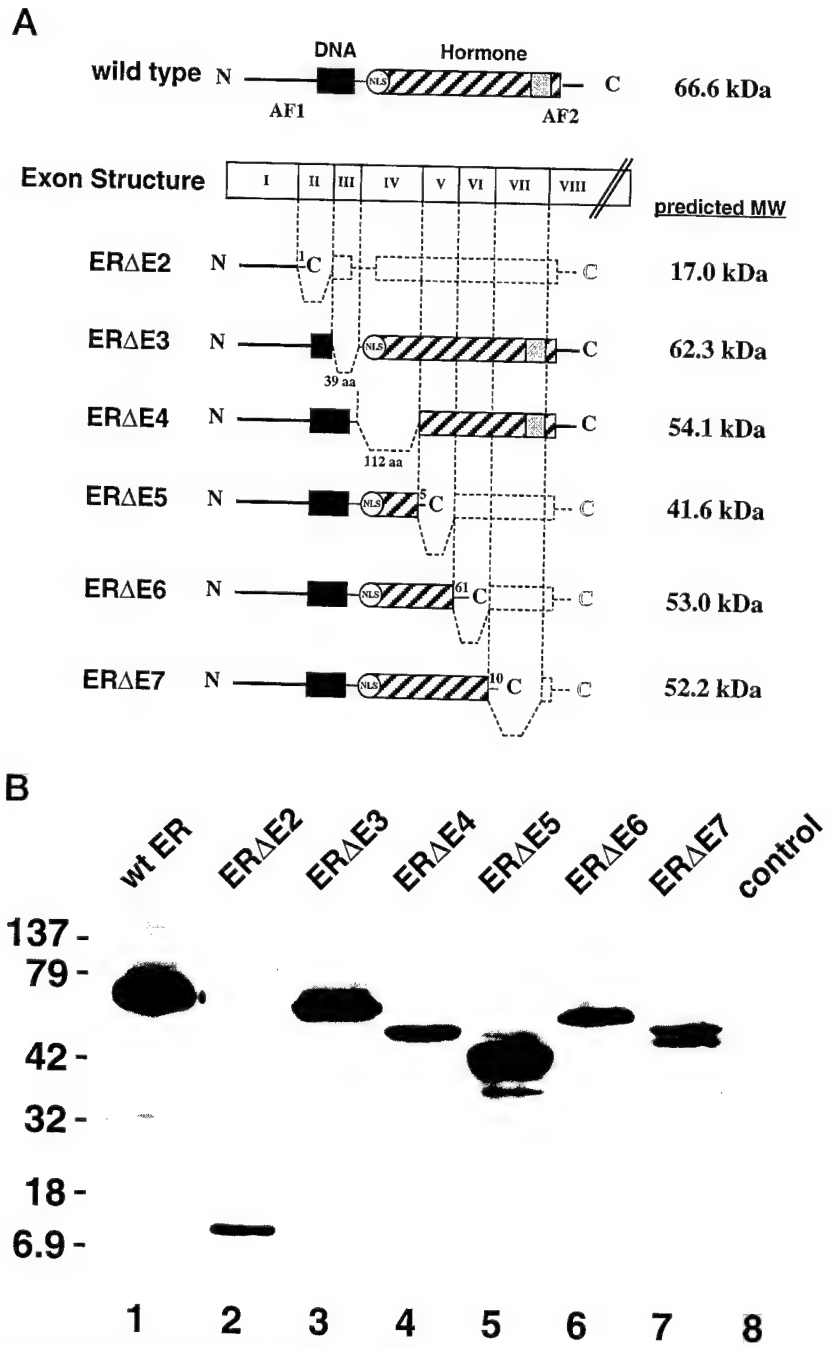

Fig. 1. Comparison of ERα mRNA Splicing Variants and wt ERα Structure

Panel A depicts the various functional domains of the receptor and the exon sequences from which they are derived. The variants are referred to by deleted exon. The size and mol wt of each variant are predicted from the translational reading frame of the sequenced cDNA clones. *Dashed lines* indicate regions of the major open reading frame of the full-length ERα protein that are missing from each variant. The nuclear localization signal is *circled*. The regions encompassing the DNA- and hormone-binding domains are marked by *darkened and hatched boxes*, respectively, where they are expressed. The AF-1 and AF-2 domains are indicated where they reside within the N and C termini, respectively, of the wild-type receptor. B, Immunoblot analysis indicates that the mol wt of each variant is consistent with its predicted translational reading frame. Samples containing 20 μg of protein from extracts of transfected Cos 7 cells were analyzed on a 10% SDS-polyacrylamide gel probed with the ERα-specific antibody, Mab-17. The figure is representative of three independent transfection experiments.

assay in which the binding of a fluorescent estrogen analog was visualized in cells cultured on cover slips. Cos7 cells transiently expressing the individual variants or wt receptor were treated with the fluorescent ligand, nitrile tetrahydrochrysene (nitrile THC) (41). Only those cultures transfected with wt ERα or ERΔE3

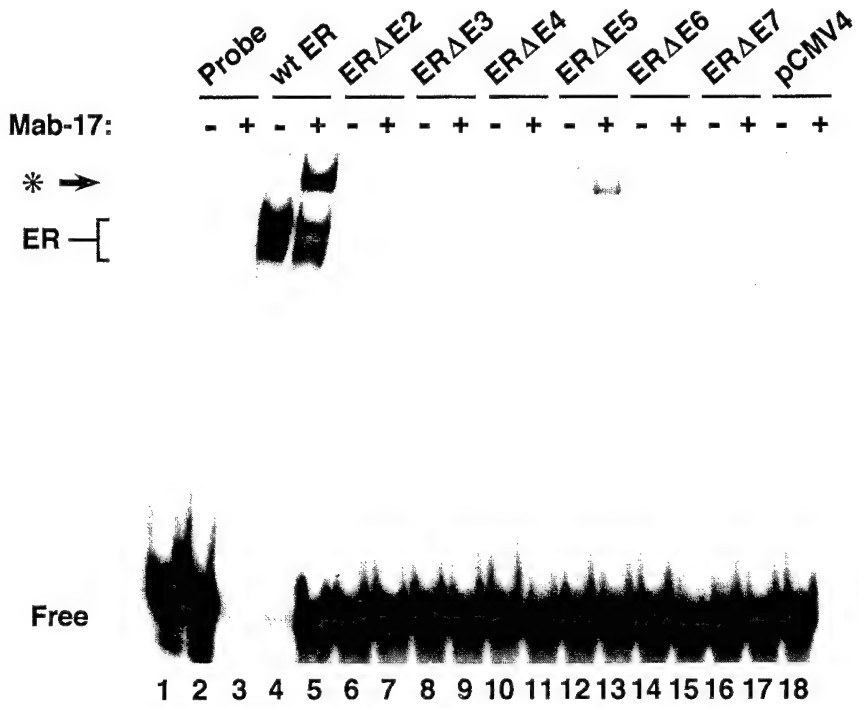

Fig. 2. Gel Mobility Shift Assay to Assess the DNA-Binding Activity of ERα mRNA Splicing Variants

A gel mobility shift assay of lysates from E_2-treated, transiently transfected Cos7 cells confirms that wt ERα binds efficiently to a ^{32}P-labeled consensus ERE. Confirmation that the indicated band represents an authentic ERα/DNA complex is provided by the ability of an ERα-specific monoclonal antibody (Mab-17) to supershift this complex (compare lane 4 with lane 3). In contrast, all of the splicing variants display strong defects in DNA binding. In this assay, which is representative of three equivalent and independent experiments, weak binding of ERΔE5 and ERΔE7 to the ERE can only be observed when their respective DNA complexes are stabilized by the addition of the ERα-specific antibody (lanes 12 and 16). Position of the antibody-supershifted complex is indicated by an *asterisk*.

were observed to stain with this ligand. In both cases, staining was localized tightly within the nucleus. This suggests that among the variants examined, wt ERα and ERΔE3 exclusively bind ligand and in both cases, ligand-bound receptors are translocated normally to the nucleus of expressing cells (Fig. 3B). With Scatchard analysis we compared the affinity of ERΔE3 and wt ERα for ^{3}H-labeled E_2. The measured dissociation constants were 0.66 nM for wt ERα and 0.79 nM for ERΔE3 (Fig. 3C).

Subcellular Localization of ERα Splicing Variants

To more carefully assess the subcellular localization of ERα splicing variants, including those that fail to bind ligand, Cos7 cells were transiently transfected with expression vectors encoding wt ERα or individual variants. These receptors were detected in transfected cells by indirect immunofluorescence staining (using the MAb-17 monoclonal antibody) and confocal microscopy. Similar to wt ERα, ERΔE3 and ERΔE5 localize to the nuclei of transfected cells, although ERΔE5 showed perinuclear as well as nuclear staining (Fig. 4). These results are consistent with the fact that both ERΔE3 and ERΔE5 retain a NLS immediately downstream of the DBD (3, 19). Subcellular localization studies have also been completed for the exon 2, 4, 6, and 7 deletion variants. Each of these proteins can be readily detected in transfected cells, but they all possess dramatic defects in nuclear targeting (Fig. 4). Nuclear targeting of wt ERα is governed, in large part, by a tripartite karyophilic signal present within exon 4 (19). Loss of this signal is therefore consistent with the cytoplasmic pattern of distribution of mutants such as ERΔE2 and ERΔE4, both of which lack protein corresponding to exon 4 sequences. Inappropriate presentation or folding of this signal must account for the defects in nuclear localization seen with ERΔE6 and ERΔE7, since the NLS is retained in these variants. Based on their subcellular distribution, we would predict that only ERΔE3 and ERΔE5, like wt ERα, would have the potential to exert nuclear effects, such as modulating gene transcription. Furthermore, the inability of the cytoplasmic variants ERΔE2, ERΔE4, ERΔE6, and ERΔE7 to dimerize with wt ERα predicts that their subcellular distribution will not be influenced by coexpression with the nuclear isoforms (wt ERα, ERΔE3, and ERΔE5). For ERΔE4, this was confirmed with a cotransport assay using a dimerization-competent ERα (data not

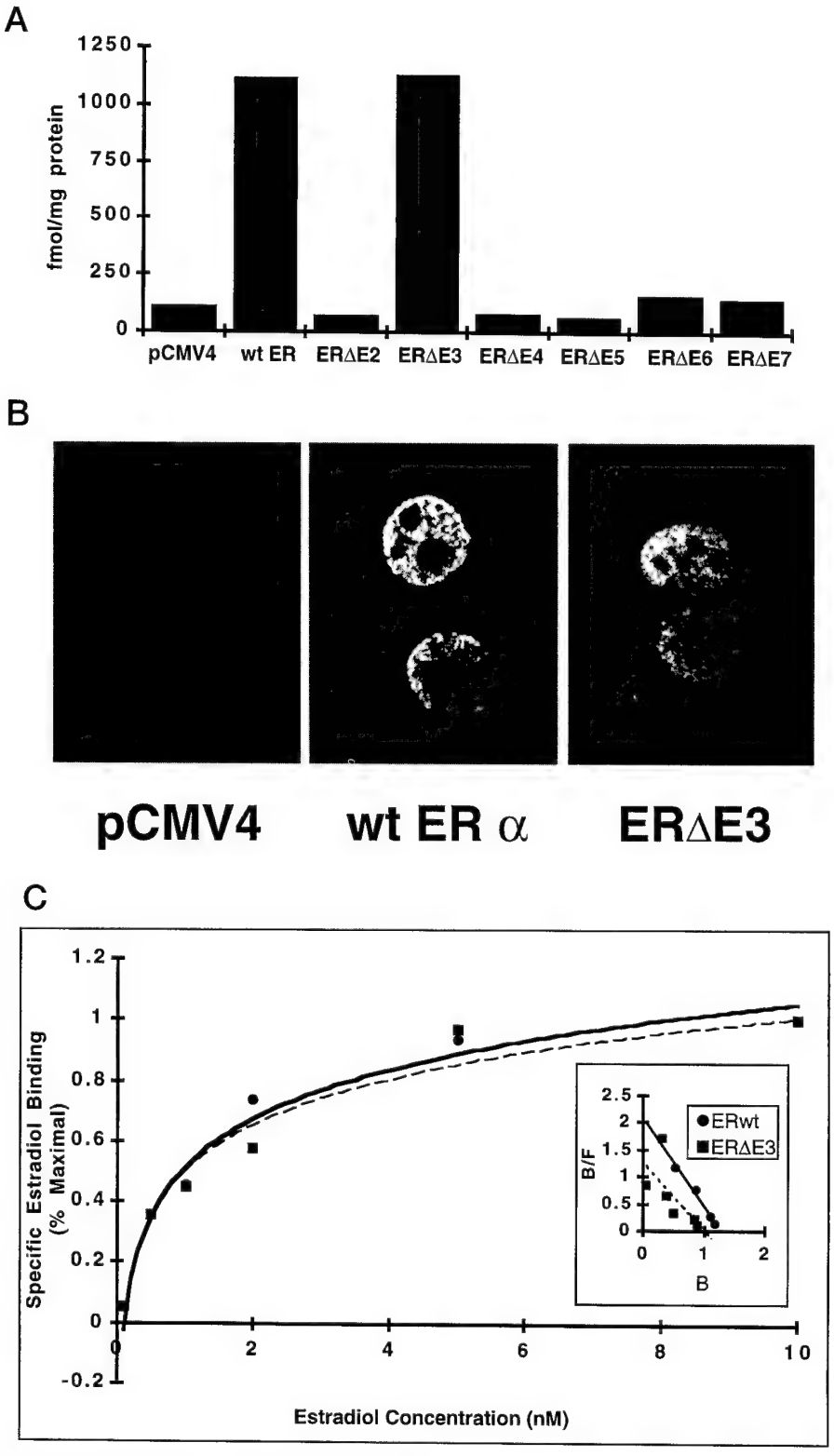

Fig. 3. Ligand Binding of ERα and ERα mRNA Splicing Variants

A, Ligand-binding capacity was assessed by measuring the specific association of 10 nM ^{3}H-17β-E_2 with wt ERα and its splicing variants expressed in Cos7 cells. Only wt ERα and the ERΔE3 variant demonstrate specific binding of E_2. B, Confirming the ligand binding results, ERΔE3 and wt ERα are the only isoforms of the receptor observed to display specific cell staining using a fluorescent estrogen analog (nitrile THC) to treat transiently transfected Cos7 cells. C, Scatchard analysis shows that wt ERα and ERΔE3 have similar affinities for 17β-estradiol.

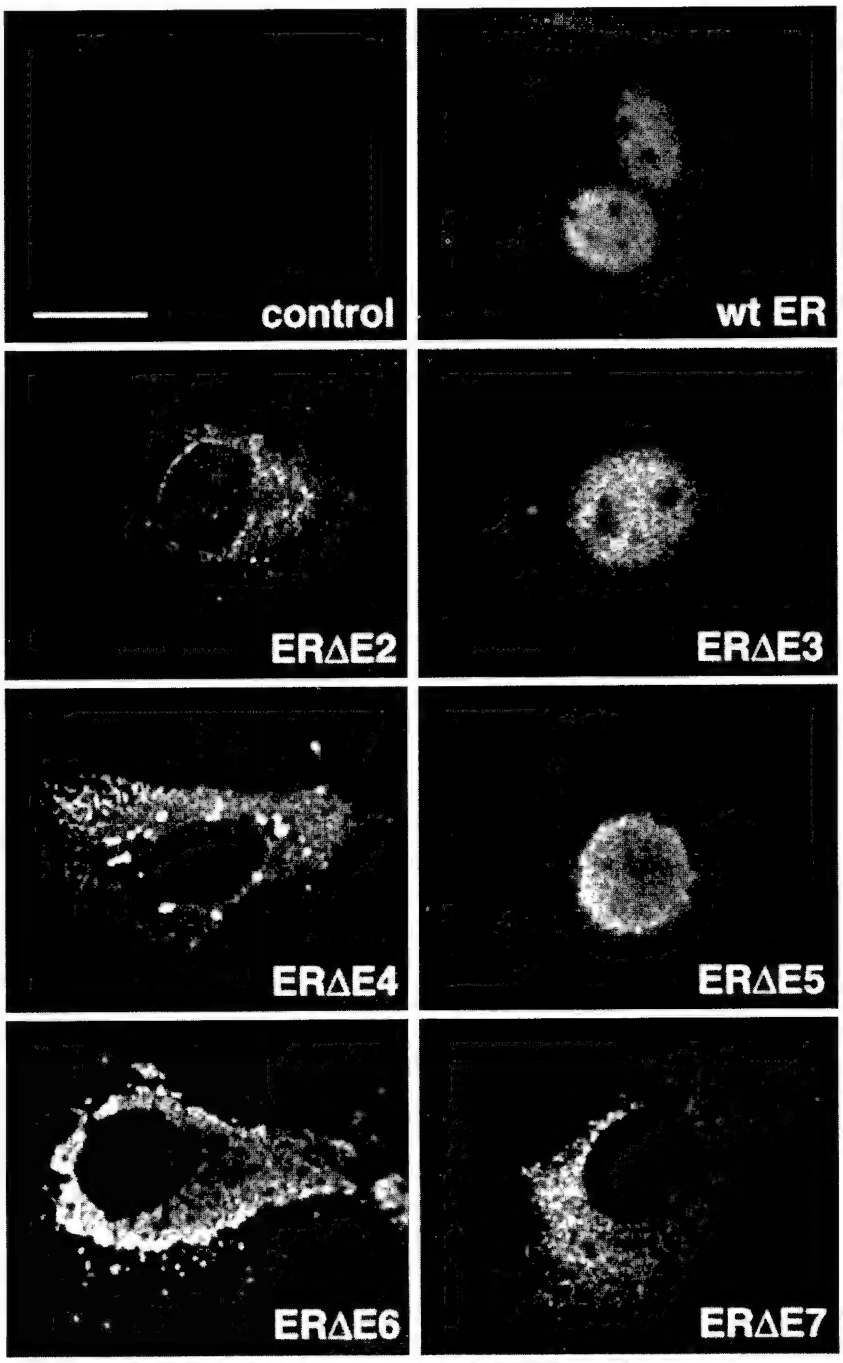

Fig. 4. Localization of ERα and ERα Splicing Variants by Confocal Microscopy

Receptor isoforms were detected by immunofluorescence staining with an ERα-specific monoclonal antibody (Mab-17) and a rhodamine-conjugated secondary antibody. The *upper left panel* shows a representative field from control cells transfected with empty expression vector (pCMV4) displaying minimal nonspecific background. Specific immunoreactivity can be observed in all other frames. Cells expressing wt ERα, ERΔE3, and ERΔE5 demonstrate strong nuclear staining in confocal sections. ERΔE2, ERΔE4, ERΔE6, and ERΔE7 are predominately localized to the cytoplasm. *Bar*, 10 μm.

shown). It is worth noting, moreover, that the existence of translationally stable, cytoplasmic splicing variants such as these may provide an explanation for cytoplasmic staining that is commonly observed during immunohistochemical analysis of breast biopsy specimens to assess ERα status.

Characterization of the Transactivation Function of ERα Splicing Variants on the Vitellogenin ERE

HeLa cell cotransfection experiments designed to assess the transcriptional activity of individually expressed ERα splicing variants have failed to demon-

strate any significant ability of variant receptors to support gene activation through an ERE, with the possible exception of the ERΔE5 variant, which is reported to display a low level of constitutive transcriptional activity on an ERE-driven reporter in some, but not all, cell types examined (23, 25, 28). In our hands none of the variants were effective transcriptional activators of an ERE-containing promoter. ERΔE5 repeatedly showed only modest constitutive activity (~5% of wt ERα induction) on an ERE-directed reporter plasmid cotransfected into HeLa cells (see Fig. 7, *inset*).

It is important to recognize that the tissues and cell lines that express these variants also express wt ERα. We have previously reported that the ERΔE3 variant acts as a dominant negative mutant when it is coexpressed with wt ERα in HeLa cells treated with E_2 (39). In the human breast epithelial cell line HMT-3522S1, ERΔE5 has also been reported to disrupt transactivation by agonist-bound wt ERα of an ERE reporter gene (38). To clarify whether this is a function unique to these variants, we completed a series of experiments to test whether the remaining exon-skipped ERα variants also support transcriptional inhibitory effects. When examined in a HeLa cell cotransfection assay in which the expression of pERE-TK-CAT was driven by E_2-bound wt ERα, a 5-fold molar excess of any of the splicing variants lacking exons 2, 4, 6, or 7 failed to inhibit the E_2-dependent induction of chloramphenicol acetyltransferase (CAT) gene expression by intact receptor (data not shown). In agreement with previously published results, the ERΔE3 and ERΔE5 variants both demonstrated a dominant inhibitory activity at all molar ratios tested (Fig. 5). With the caveat that equal amounts of plasmid DNA support similar levels of variant receptor expression (see Fig. 1B), it appears that ERΔE3 and ERΔE5 are approximately equivalent in their inhibitory activity in HeLa cells.

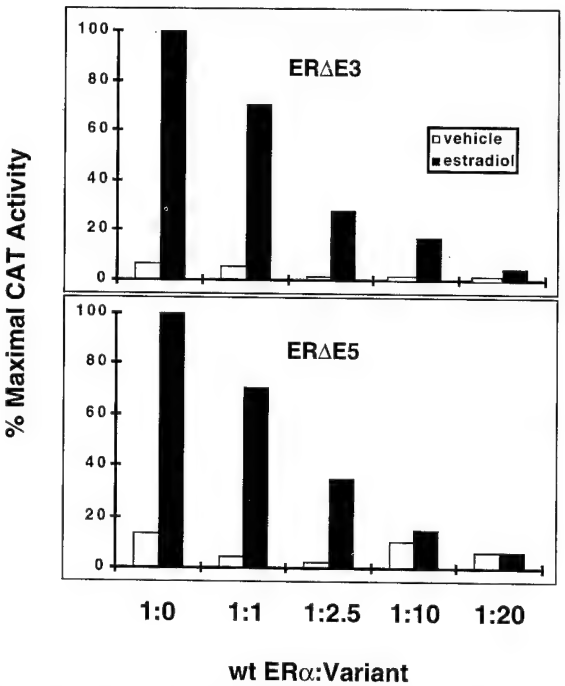

Fig. 5. ERΔE3 and ERΔE5 Inhibit Transactivation of a Consensus ERE Reporter by wt ERα in a Dose-Related Fashion

HeLa cells were cotransfected with 16 μg of pERE-TK-CAT reporter gene, 1 μg of wt ERα expression vector, and increasing amounts of expression vectors for ERΔE3 or ERΔE5 (from 0–20 μg). The ratios of wt ERα to variant expression plasmid used in each transfection are indicated. The total amount of DNA in each transfection was held constant with the addition of empty expression vector, pCMV4. Reporter gene expression was normalized by measuring CAT activity in aliquots representing 100 μg of soluble protein.

Dimerization and Coactivator Binding Properties of ERΔE3 and ERΔE5

We next questioned whether a direct interaction between the variants and factors responsible for ERE-directed transcription might explain the inhibitory effects of ERΔE3 and ERΔE5 on wt ERα activity; specifically, we tested for a direct interaction of ERΔE3 and ERΔE5 with wt ERα and steroid receptor coactivator-1e (SRC-1e). The C terminus of wt ERα and fragments of the SRC-1e protein were expressed as fusion proteins with glutathione *S*-transferase (GST) and attached to glutathione-Sepharose beads. Binding assays with GST fused to the C terminus of wt ERα (GST-AF2) and ^{35}S-methionine labeled *in vitro* translated receptor demonstrate that ligand-bound wt ERα and ERΔE3, but not ERΔE5, dimerize with the LBD of ERα in solution (Fig. 6A). In experiments with GST-SRC-1e fragments, both ERΔE3 and ERΔE5 were observed to bind to regions of the coactivator that also bind wt ERα (Fig. 6B). ERΔE3 and wt ERα bind the SRC-1e fragments comprising amino acids 570–780 and 989-1240 and do so only in the presence of E_2. In contrast, binding of ERΔE5 to the 989-1240 amino acid fragment is constitutive (Fig. 6B).

ERΔE3 Is a Positive Regulator of Gene Expression on an AP-1 Reporter

The results presented above indicate that ERα splicing variants are either inactive (ERΔE2, ERΔE4, ERΔE6, and ERΔE7) or largely inhibitory (ERΔE3 and ERΔE5) in their effect on reporter constructs that contain a consensus ERE. Recent reports suggest that wt ERα is also able to transactivate genes whose promoters do not contain an obvious ERE. In particular are promoters for the human collagenase, chicken IGF-I and ovalbumin genes that are regulated by wt ERα and contain a critical AP-1 element (29, 31, 32). Mutational analysis revealed that the DBD was not required for ERα-dependent expression of these genes. Clearly, the mechanisms of ERα transcriptional activity and DNA targeting are complicated by these reports. We propose that to assess the transactivating potential of ERα variants, the promoter focus must be expanded to include promoters that contain noncanonical regu-

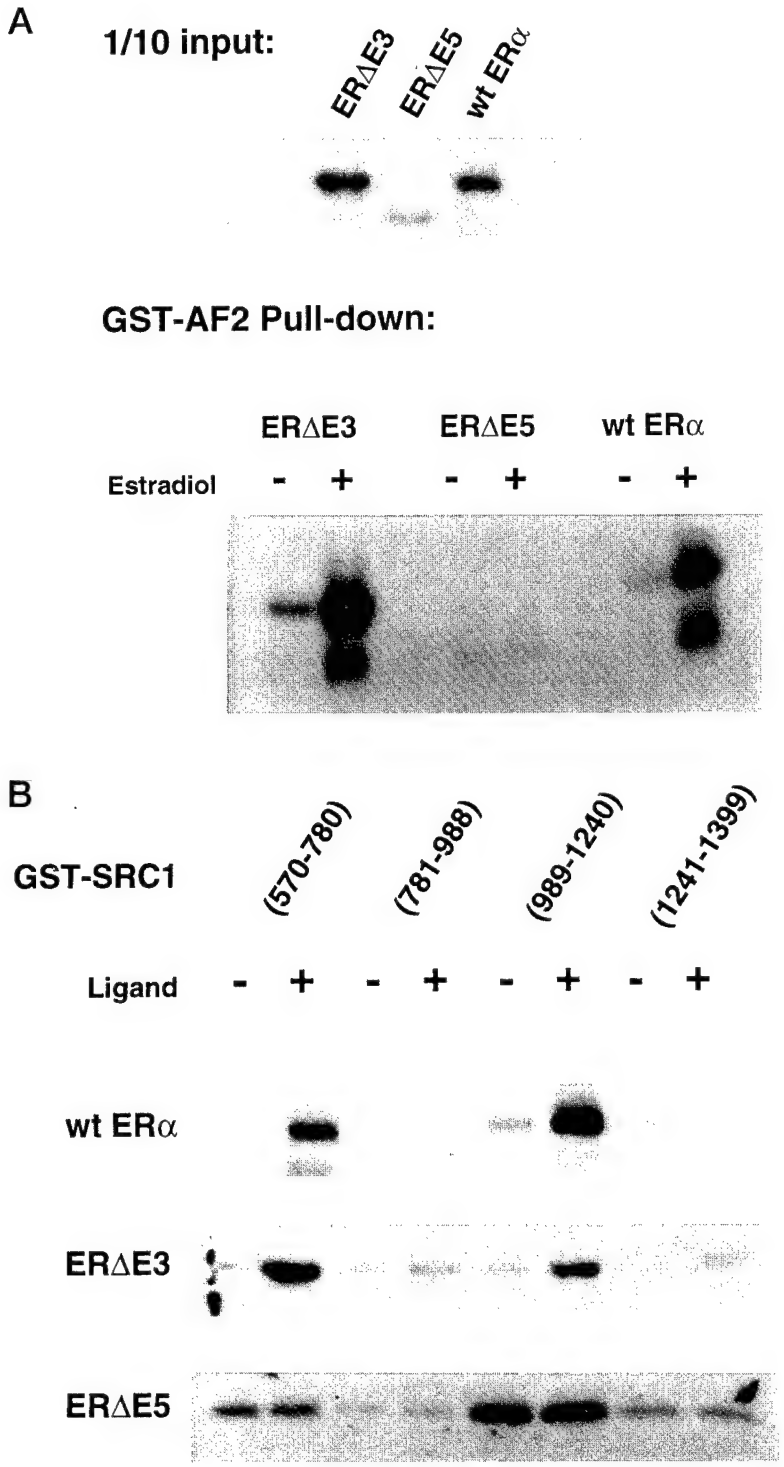

Fig. 6. *In Vitro* Binding of Radiolabeled Receptor Variants with GST-Fused wt ERα and SRC-1e Fragments

A, An autoradiograph showing *in vitro* translated, ^{35}S-methionine-labeled wt ERα and ERΔE3 retained by the AF2 domain of wt ERα receptor fused to GST (GST-AF2) and complexed to glutathione-Sepharose beads. Dimer formation requires the presence of 2.5 μM E_2. ERΔE5 does not bind to the AF2 domain of ER. *Top panel* represents 10% of the radiolabeled input. B, In the presence of ligand, wt ERα and ERΔE3 bind to SRC-1e fragments comprising amino acids 570–780 and 989–1240. The *lower panel* shows a strong constitutive interaction between radiolabeled ERΔE5 and SRC-1e fragment (989–1240).

latory elements. These thoughts prompted us to test the activity of the ERα variants on the ovalbumin promoter that contains a complex hormone response element. We performed cotransfection experiments in HeLa cells using vectors expressing wt ERα or the exon-skipped variants ERΔE2 through ERΔE7 and a CAT reporter gene construct, pOvalb-CAT, driven by a fragment of the ovalbumin promoter (−1342 to +7 relative to the transcription start site) described to encompass much of the regulatory sequence of this gene (35, 42). Results from these experiments indicate that both wt ERα and ERΔE3 support inducible gene expression from the ovalbumin promoter (Fig. 7) and that all of the remaining single exon-skipped variants are transcriptionally inactive on this reporter construct (data not shown). For wt ERα, this corroborates previously published reports (29, 35). Maximal activity was measured in cultures treated with both phorbol 12-myristate, 13-acetate (PMA, a phorbol ester) and E_2, where a 16-fold induction was observed. Like wt ERα, ERΔE3 reproducibly induced this reporter, despite its lack of an intact DBD. While the induction shown in Fig. 7 for ERΔE3 (averaging 9-fold) is less than that supported by wt ERα, the activity of ERΔE3 equaled and occasionally exceeded that of the intact receptor in several individual experiments, confirming that this variant can be a potent inducer of transcription. In both cases cotreatment with PMA and E_2 is highly synergistic as E_2 treatment alone has no significant effect, and PMA treatment alone supports only modest induction for wt ERα (2.5-fold relative to vehicle control, $P < 0.001$). Tamoxifen treatment of wt ERα- or ERΔE3-transfected cultures, either alone or together with phorbol ester, had no significant effect on pOvalb-CAT expression. This contrasts with the stimulatory activity of tamoxifen observed on other AP-1 containing estrogen-responsive reporter genes (32). In control cells transfected with an empty CMV expression vector, treatment with PMA yielded negligible reporter gene activity. This suggests that, in the absence of ERα, activation of endogenous AP-1 alone is not an effective inducer of transcription from the ovalbumin promoter in these cells. To confirm that wt ERα and ERΔE3 cooperate with AP-1 factors to regulate transactivation of the ovalbumin promoter, we measured pOvalb-CAT expression in HeLa cells cotransfected with both a receptor isoform and cJun. Transcriptional activity of wt ERα and ERΔE3 supported by PMA and E_2 cotreatment was enhanced by cJun overexpression. While the presence of endogenous AP-1 tended to obscure the synergism between cJun and wt ERα in this system, the combined effects of these transcription factors were slightly more than additive. A greater than additive activation was also

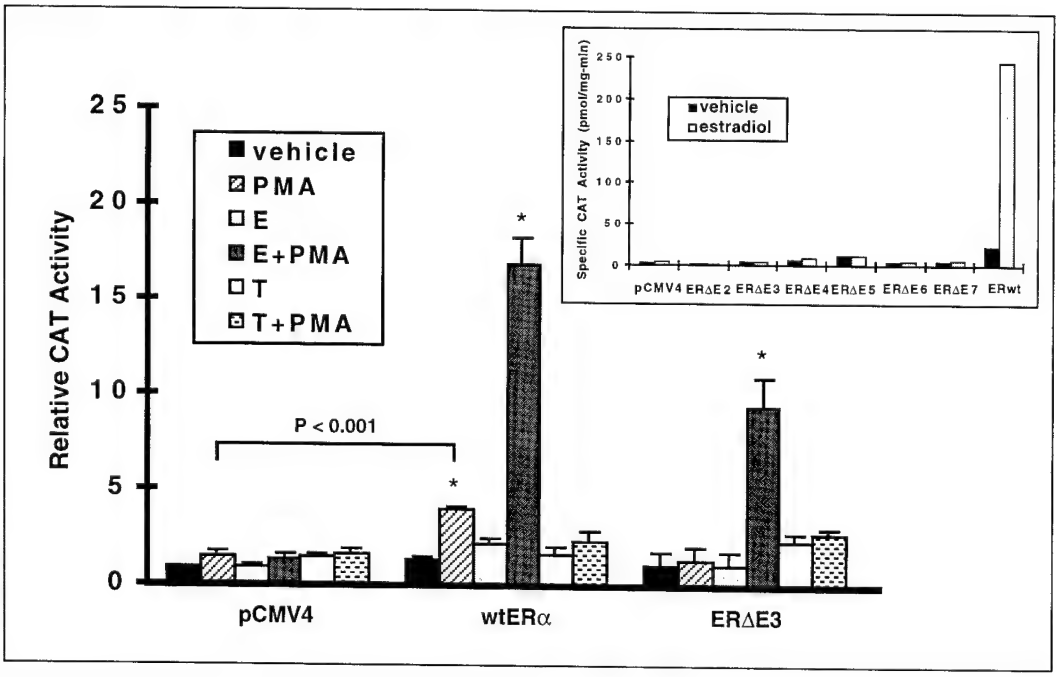

Fig. 7. Transcriptional Activity of wt ERα and Receptor Variants

Only ERΔE3 supports CAT gene expression from the ovalbumin promoter, similar to wt ERα. HeLa cells were cotransfected with 16 μg of reporter (pOvalb-CAT) and 0.5 μg of the indicated ERα expression vector followed by 24 h of hormone treatment in the presence of 5% charcoal-treated calf serum. Cultures were treated as indicated: vehicle control; 2×10^{-8} M PMA; 10^{-8} M 17β-estradiol (E); E + PMA; and 10^{-7} M tamoxifen (T) or T + PMA. CAT assays were normalized for equal amounts of protein. Values are expressed relative to vehicle-treated empty expression vector, pCMV4. *Error bars* represent the SEM of three independent experiments (*, $P < 0.001$). As shown in the *inset*, aside from wt ERα itself, none of the ERα splicing variants tested are strong activators of the ERE-driven CAT reporter gene (pERE-TK-CAT) in HeLa cells.

observed when cJun and ERΔE3 were coexpressed (Fig. 8). Exogenous cJun alone elicited only a modest response to PMA and E_2 treatment. These observations, combined with the dual requirement for activating both AP-1 and ERα, strongly suggest that these factors are acting cooperatively on the ovalbumin promoter.

DISCUSSION

Our efforts to functionally characterize exon-skipped ERα mRNA splicing variants have identified two receptor isoforms that possess the ability to modulate estrogen signaling on genes that are targeted by the ER. Although their protein structure is significantly altered, the ERΔE3 and ERΔE5 splicing variants retain many of the activities attributed to the full-length receptor. Loss of exon 3 results in a receptor protein with an internal deletion that lacks a major portion of the DBD and therefore prevents ERΔE3 from binding to a consensus ERE, as confirmed by gel mobility shift analysis. However, ERΔE3 retains the LBD and NLS, thereby allowing it to bind hormone with an affinity similar to wt ERα and translocate to the nucleus. The deletion of exon 5 causes a frame-shift mutation and results in a C-terminally truncated form of the receptor. Loss of the LBD predictably renders ERΔE5 unable to bind E_2. Nonetheless, ERΔE5 still retains the NLS, and immunofluorescence analysis shows nuclear staining in Cos7 cells transfected with this variant.

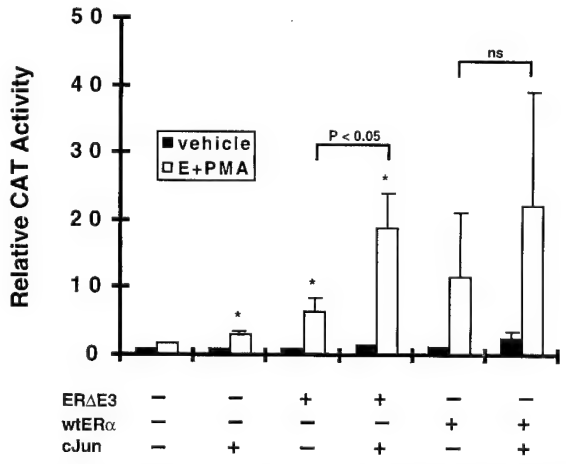

Fig. 8. Effect of cJun Overexpression on wt ERα and ERΔE3 Activation of the Ovalbumin Promoter

Cotransfection of a cJun expression vector enhances both wt ERα and ERΔE3 transactivation of the pOvalb-CAT reporter gene in HeLa cells cotreated with vehicle or 2×10^{-8} M PMA and 10^{-8} M 17β-estradiol (E + PMA). The addition of cJun increased the induced activity of the ovalbumin promoter approximately 2-fold relative to either receptor isoform alone. CAT assays were normalized for equal amounts of protein. Values are expressed relative to vehicle-treated empty expression vector, pCMV4. *Error bars* represent the SEM of at least four independent experiments (*, $P < 0.05$).

Rather than serving to stimulate transcription on a consensus ERE, results from transient transfection experiments in HeLa cells that combine either ERΔE3 or ERΔE5 with wt ERα and an ERE-driven reporter gene indicate that these isoforms actually function to inhibit transcriptional activation by wt ERα. These observations agree with our previous results and with those reported by others from similar experiments using HMT-3522S1 cells (38, 39). A 70% inhibition of transcriptional activation by E_2-liganded wt ERα on an ERE-driven CAT reporter gene was observed in HeLa cells when ERΔE3 and wt ERα expression vectors were cotransfected at a ratio of 5:1 (39). In the ER-negative cell line HMT-3522S1, coexpression of an equal amount of ERΔE5 significantly inhibited stimulation of an ERE reporter construct by wt ERα (38). Increasing the ratio of transfected variant to wt ERα demonstrates that the repression of wt ERα by ERΔE3 and ERΔE5 is dose-related and becomes nearly complete when the variants are present in sufficient excess (38, 39). This observation has physiological significance in the case of breast tumor cells that predominantly express one of these splicing variants (12, 22). Castles *et al.* (22) report that ERΔE5 is the major ER transcript in BT-20 and MDA MB 330 breast tumor cell lines. In BT-20 cells the ERΔE5 variant comprises 68% of the ER mRNA population while wt ERα measures 8%. Studies by Erenburg *et al.* (43) indicate that, while ERΔE3 tends to be underrepresented in breast tumors and tumor cell lines, it typically constitutes 50% or more of ERα mRNA in both stromal fibroblasts and epithelial cells isolated from reduction mammoplasty specimens. These authors further demonstrated that stable overexpression of ERΔE3 in MCF-7 cells to levels seen in normal mammary epithelial cells dramatically reduced the expression and estrogen inducibility of endogenous pS2 mRNA, as well as reducing their anchorage-independent growth and *in vivo* invasiveness (43).

The dominant negative character of ERΔE3 and ERΔE5 suggests that, like wt ERα, these variants are able to interact with at least one component of the ERE-directed transcription complex in a manner that disrupts positive gene regulation by wt ERα. Based on gel mobility shift assay analysis, it is unlikely that transcriptional interference by these variants involves binding to an ERE to the exclusion of wt ERα. Our DNA binding analysis indicates that ERΔE5 can bind only weakly to DNA, and only when the formation of this complex is stabilized by the addition of a bivalent antibody. The role of the antibody in this case is presumably to substitute for the missing dimerization interface and to tether receptor subunits together in a form more able to interact with DNA. DNA binding by ERΔE7 similarly requires the addition of antibody, but this binding is even less efficient than binding by ERΔE5. Interestingly, a correlation exists among the ERα variants between their ability to translocate to the

nucleus and their transcriptional inhibitory effect on wt ERα activity in mammalian cells. As of yet, no clear function has been established for the ERΔE7 variant in mammalian cells, despite an earlier report that ERΔE7 is a dominant inhibitor of wt ERα function in yeast (44). This is noteworthy since a number of quantitative studies have indicated that, as a rule, ERΔE7 represents the most abundant of the ERα splicing variants in breast tumors (summarized in Ref. 15).

We have previously reported that although ERΔE3 is unable to bind to an ERE itself, it can prevent wt ERα from binding to DNA (39). That ERΔE3 inhibits both DNA complex formation and transactivation by wt ERα suggests that the potential targets of interaction by ERΔE3 may include protein-protein contacts with wt ERα itself or interactions with nuclear receptor coactivators or other receptor-associated factors. ERα function may be disrupted when ERΔE3, which lacks the DBD but retains the hormone-inducible dimerization domain, forms mixed dimers with wt ERα that are inefficient at binding stably to DNA. We are able to show that, in the presence of E_2, ERΔE3 (but not ERΔE5) can form a stable complex with the LBD of ERα fused to GST attached to glutathione-Sepharose beads. This is consistent with a model for direct inhibition of the DNA binding activity of full-length receptor by ERΔE3. Experiments using fragments from SRC-1e fused to GST indicate that both ERΔE3 and ERΔE5 can bind a nuclear receptor coactivator. Similar to the pattern of wt ERα interaction with SRC-1e, *in vitro* translated ERΔE3 is able to associate in an E_2-dependent manner with two regions of the steroid coactivator SRC-1e (amino acids 570–780 and 989-1240). This agrees with previous reports that also describe three conserved nuclear receptor-binding motifs (LXXLL) within the 570–780 amino acid region and a distinct site for AF-1 interaction within the 989-1240 amino acid fragment (17, 45). A site for SRC-1 interaction within ERα corresponds with the AF2 domain (46), a region that is retained in the ERΔE3 variant. Isoforms of SRC-1 are potent enhancers of agonist-bound ERα and are required for its full transcriptional activity (47, 48). Transfection experiments in E_2-treated HeLa cell cultures demonstrate that coexpression of mutants containing the C terminus of ERα can attenuate ERα-dependent gene expression and that this decreased activity can be overcome with simultaneous overexpression of the SRC-1-related coactivator transcriptional intermediary factor 2 (TIF2) (49). These results suggest that coactivators are limiting factors for which the receptors are competing and that ERΔE3, like wt ERα, is a target for SRC-1 binding.

In a surprising result from cotransfection studies using engineered mutants of ERα, maximal expression of an ERE-containing reporter gene could be observed when SRC-1 was transfected simultaneously with separate N- and C-terminal fragments of ERα, containing the AF-1/DBD and the LBD/AF2 regions, respectively (50). These results suggest that separate AF1- and AF2-containing ERα polypeptides can interact in a transcriptionally productive manner, provided they are brought together by SRC-1. Furthermore, they provide an initial indication that SRC-1 interacts separately and perhaps directly with both the AF1 and AF2 domains. More support for this notion is provided by our observations that ERΔE5 binds to the SRC-1e amino acid fragment 989-1240 in solution. These results suggests the possibility that the inhibitory function of ERΔE5, which itself is relatively inefficient at binding DNA or activating transcription through an ERE, most likely results from competition with wt ERα for interaction with SRC-1 or other cellular factors.

The most compelling evidence that some of the ERα mRNA splicing variants may indeed be transcriptionally active is seen in transfection experiments involving ERΔE3 and reporter gene constructs containing a nonconsensus hormone-regulatory element. Recently, a novel mechanism for mediation of an estrogen response has been reported to involve AP-1-directed regulation of transcription by ERα (29–32). AP-1 and its isoforms represent a family of nearly ubiquitous transcription factors whose activity is crucial for the efficient expression of a wide variety of genes. As an important downstream target for the mitogen-activated protein kinase (MAPK)- and Jun kinase signaling cascades, AP-1 is a central player in mediating the effects of serum and growth factors on cellular proliferation (33, 34). A variety of estrogen-responsive genes have been described that lack a palindromic ERE, but instead contain one or more consensus AP-1 elements that often occur near a degenerate ERE or ERE half-site (29, 31, 32). It should be noted that these imperfect EREs may in some cases serve dual function as cryptic AP-1 response elements whose consensus sequence (5′-TGAG/CTCA-3′) bears superficial similarity to an ERE half-site (5′-GGTCA-3′ or 5′-TGACC-3′).

An important observation from the analysis of genes regulated by noncanonical EREs is that the structure-activity requirements for activation by ERα (both for the ligand and for the receptor) are different than those for transcriptional activation through a palindromic ERE. Using a region of the collagenase gene promoter (−73/+63) that lacks an ERE but harbors an essential AP-1 element, Kushner and co-workers (32) demonstrated that a DBD-deleted mutant of ERα was significantly more effective at supporting E_2-induced reporter gene expression than wt ERα in transfected HeLa cells. Similar to collagenase gene expression, ERα-dependent activation of the chicken ovalbumin promoter, which lacks a palindromic ERE, does not require an intact DBD (29). Furthermore, an ERE half-site was determined to be the site for synergistic regulation of ovalbumin gene expression by AP-1 and ERα (29). Our studies involving cotransfection of an ERα splice variant with the ovalbumin promoter construct, pOvalb-CAT, agree with these findings. Results from Fig. 7 demonstrate that, compared with mock-transfected HeLa control cultures, pOvalb-CAT is strongly activated by either wt ERα or ERΔE3. A breakdown of treatments indicates that maximal activity of

both wt ERα and ERΔE3 clearly requires E_2 in addition to an AP-1 activator. *In vitro* assays demonstrate an interaction between cJun and the N terminus of ERα fused to GST (32). Additional evidence suggesting that ERΔE3 and wt ERα cooperate with activated AP-1 to maximally transactivate the ovalbumin promoter is provided by our observation that receptor activity is enhanced by simultaneous cJun overexpression. In our studies tamoxifen treatment had little or no effect on the activity of wt ERα or ERΔE3, either with or without PMA cotreatment. This contrasts with results observed when wt ERα was cotransfected with a collagenase reporter construct in HeLa cells, where tamoxifen supported a significant induction of reporter gene expression (32).

Our transfection results show that, while several of the ERα splicing variants are functionally incapacitated by their deletions, two of the variants clearly retain significant transcriptional activity. For the ERΔE3 and ERΔE5 variants, this activity is quite complex. Both of these variants represent stable receptor isoforms that, like the full-length receptor, localize efficiently to the nucleus where they can interact with the transcription apparatus. However, when acting through a consensus ERE, these variants completely lack (ERΔE3) or show only weak (ERΔE5) transcriptional stimulatory activity, consistent with their poor DNA binding ability. On the contrary, both variants serve to blunt the ability of coexpressed wt ERα to promote transcription of ERE-containing genes. At the same time, the ability of ERΔE3 (and presumably also ERΔE5) to interact productively with nuclear receptor coactivators or other transcription factors gives these ERα splicing variants the potential to stimulate or otherwise modulate gene expression through nonconsensus hormone response elements that are targeted by AP-1 motifs or other DNA-binding sites. We have clearly shown this to be true for ERΔE3 and the chicken ovalbumin promoter and believe that this is also likely to be true for many other genes, such as those encoding collagenase, cathepsin D, IGF-I, transforming growth factor-β, c-fos, heat shock protein-27, and retinoic acid receptor-α, all of which lack an obvious ERE and yet still respond to estrogen. In this respect, ERα splicing variants may actually serve to redirect transcription away from ERE-containing genes to genes such as these that appear to be regulated nonclassically by estrogens.

MATERIALS AND METHODS

Expression Vectors

Plasmids for ERα mRNA splicing variant cDNAs were generated as derivatives of pCMV4 (51) and pcDNA3.1 (Invitrogen, San Diego, CA), which support high levels of receptor expression in HeLa and Cos7 cell lines (41). Plasmids expressing ERΔE4, ERΔE5, and ERΔE6 were generated using synthetic oligonucleotides to construct the variant splice junctions within an otherwise wt ERα cDNA expression plasmid. The remaining plasmids were constructed with the use of flanking restriction sites to shuttle cloned cDNAs (39) into the appropriate expression vectors. Mouse cJun cDNA cloned into the pCMV2 expression vector was provided by L. McCabe (Michigan State University, East Lansing, MI).

Cell Culture, Transfection, and CAT Assays

Cos7 and Hela cells were grown in phenol red-free DMEM supplemented with 10% calf-serum, 5 mM HEPES (pH 7.4), 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were transfected by the $CaPO_4$ method, as previously described (52). HeLa cells (~2×10^{-6} cells per 100-mm dish) were transfected with 1 μg of the indicated ERα expression plasmid, 2 μg of the cJun expression plasmid (where indicated) and 16 μg of the estrogen-responsive reporter plasmid, pERE-TK-CAT (53) or pOvalb-CAT (a reporter gene construct containing −1342 to +7 bp of the chicken ovalbumin promoter relative to its transcription start site) (42). Calf thymus DNA (10 μg) was added as carrier. After overnight incubation with DNA, culture medium was replaced with 5% charcoal-treated serum-supplemented DMEM containing the indicated hormones. After a 24-h incubation, cells were harvested and CAT assays were performed as previously described (54) using 100 μg protein. Quantification of CAT activities was performed by phosphorimage analysis of thin layer chromatographs (ImageQuaNT, Molecular Dynamics, Inc., Sunnyvale, CA). For experiments involving biochemical or cytochemical analysis of ERα variants, Cos7 cells were similarly transfected with 10 μg of the indicated expression plasmid and 10 μg of calf thymus carrier DNA. After overnight exposure to DNA, cells were cultured for 48 h in 10% calf serum-supplemented DMEM. All experiments involving extracts from transfected cells were normalized with respect to protein, as measured using the method of Lowry *et al.* (55). Two-way ANOVA and comparison with Student's *t* test were used to assess statistical differences between groups. Statistical significance was set at the $P < 0.05$ or $P < 0.001$ level as indicated in Figs. 7 and 8.

E_2 Binding Analysis

Ligand-binding assays were performed as previously described (40). Whole-cell extracts were prepared from transfected Cos7 cells that were resuspended and sonicated in extraction buffer (20 mM HEPES, pH 7.4, 20% glycerol, 0.4 M KCl, 1 mM $MgCl_2$) supplemented immediately before use with protease inhibitors (0.05 mg/ml each of chymostatin, trypsin inhibitor, antipain, leupeptin, aprotinin, and pepstatin). Aliquots containing 200 μg of protein were incubated overnight at 4 C with various concentrations (0.1 nM–10 nM) of ^{3}H-labeled E_2 (NEN Life Science Products, Boston, MA) in the presence or absence of a 200-fold molar excess of unlabeled E_2. Free ligand was separated from bound ligand by treatment with dextran-coated charcoal. For determination of equilibrium binding constants, these data were plotted according to the method of Scatchard (56).

DNA Binding Assays

DNA binding assays were performed as previously described (40). Aliquots containing 30 μg of protein from extracts prepared as above from transfected Cos7 cells were preincubated for 15 min at room temperature in 10 μl binding buffer [10 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 1 mM dithiothreitol, and 20% glycerol] containing 1 μg poly (dI-dC), with or without 1 μl of added human ER-specific monoclonal antibody (Mab-17), generated as described by Neff *et al.* (40). Approximately 6 fmol (40,000 cpm) of a ^{32}P-labeled double-stranded ERE oligonucleotide (39) were added to the samples and incubated for 30 min at room temperature, followed by an addi-

tional 5-min incubation at 4 C. Samples were then loaded on a preelectrophoresed nondenaturing 5% polyacrylamide gel that was run in 0.5 × Tris-Borate-EDTA at 275 V for 2 h. The gel was dried and exposed for autoradiography.

Immunoblot Analysis

Discontinuous 12% SDS-PAGE was carried out as previously described (57). After electrophoresis of 30 μg of whole-cell protein from extracts of transfected Cos7 cells, proteins were electrophoretically transferred to nitrocellulose filters with a Trans Blot apparatus (Bio-Rad Laboratories, Inc. Richmond, CA) using the procedure of Erickson *et al.* (58). Immunoblots were probed with the ER-specific monoclonal antibody, Mab-17, obtained from a hybridoma culture supernate that was diluted with an equal volume of PBS (40). Immunoreactive protein was visualized by enhanced chemiluminescence using a horseradish peroxidase-conjugated goat antimouse IgG, following manufacturer's instructions (Amersham Pharmacia Biotech, Arlington Heights, IL).

In Vitro Protein-Protein Interaction Assays

Variant and wt ERα receptor protein was translated in the presence of [^{35}S]methionine using the TNT Coupled Reticulocyte System (Promega Corp., Madison, WI). GST-fusion proteins were expressed in the pGEX system (Pharmacia Biotech, Uppsala, Sweden) (45, 59). Overnight cultures of transformed bacteria were diluted 1:20 and cultured for 2 h before protein expression was induced with the addition of isopropyl β-D-thiogalactoside (IPTG, 0.2 mM final concentration). Bacteria were collected by centrifugation 2 h following IPTG induction, and pellets were resuspended in 400 μl of extraction buffer supplemented with protease inhibitors. Cells were sonicated briefly, and the resulting lysates were centrifuged for 20 min at 20,000 rpm, 4 C. Protein concentrations were determined (55) and extracts were diluted to 2 μg/μl extraction buffer and stored at −70 C until binding assays were performed.

Before use in protein interaction assays, 25 μl of glutathione-Sepharose 4B beads (Pharmacia Biotech) were washed three times in 100 μl NETN [0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris (pH 8.0) 100 mM NaCl] and suspended in 100 μl NETN, 0.5% powdered milk. Washed beads were incubated with 40 μg of GST-fusion protein for 2 h, rotating at room temperature. Beads complexed with GST-fusion proteins were washed three times with 100 μl NETN, 4 C. For protein-protein interaction assays, 5 μl of *in vitro* translated receptor were added to washed complexed beads resuspended in 100 μl NETN supplemented with protease inhibitors (as above) with and without 2.5 μM E_2. After a 2-h incubation during which the samples were rotated at room temperature, the beads were pelleted and washed four times with 100 μl NETN, 4 C. Bound proteins were separated on a discontinuous 10% polyacrylamide SDS-PAGE gel (57). The gels were dried and exposed for autoradiography.

Immunohistochemical and Cytochemical Analysis

Indirect immunofluorescence analysis was performed as previously described (40) using Cos7 cells that were plated and transfected on glass cover slips. On the second day after transfection, cells were washed three times with Tris-buffered saline (TBS), fixed for 3 min in cold 95% methanol, rehydrated by three washes with TBS, and incubated 30 min at 37 C with primary antibody (Mab-17 hybridoma supernate used at a 1:10 dilution in TBS). Bound antibody was detected by staining with a rhodamine-conjugated affinity-purified goat antimouse IgG (Roche Molecular Biochemicals, Indianapolis, IN) diluted 1:2000 in TBS, and incubating for 30 min at 37 C in the presence of 0.02 μg/ml of 4′,6-diamidine-2-phenylindole dihydrochloride. Confocal images were recorded using the Odyssey system (Noran Instruments, Middleton, WI) on an Optiphot 2 Nikon (Melville, NY) microscope. Fluorescent ligand staining of transfected Cos7 cells was performed as described by Miksicek *et al.* (41) on live, whole-cell mounts treated in DMEM with 10^{-7} M nitrile THC. For these studies, cells were visualized using a Nikon UFX microscope equipped with a 100 watt mercury lamp for fluorescence excitation, and a 40 × 0.7 numerical aperture Plan objective.

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